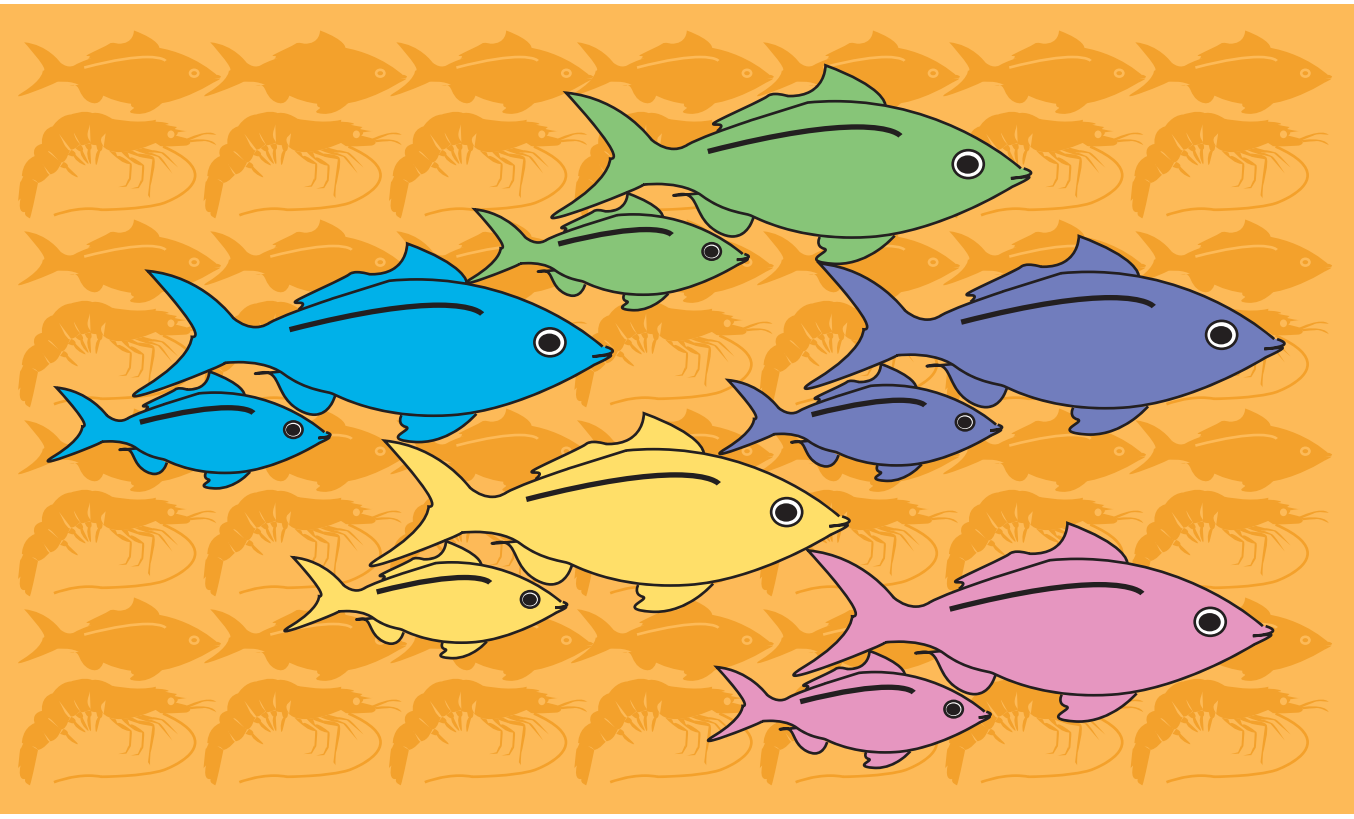
# Aquaculture Medicine and Aquatic Animal Health Management

Editors

I S Bright Singh and Y S Yadava



AQUACULTURE AUTHORITY

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**I S Bright Singh and Y S Yadava**

**Aquaculture Authority**
Ministry of Agriculture
Department of Animal Husbandry, Dairying and Fisheries
Shastri Bhavan Annexe, Chennai– 600 006, Tamil Nadu

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**National Centre for Aquatic Animal Health**
Cochin University of Science and Technology
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## FOREWORD

During the last two decades, aquaculture has grown as a viable industry that can help meet the growing protein requirements of the global population.

Though farming techniques for many fin and shellfish varieties have been standardized and aquaculture production has gone up manifold, disease prevention and treatment practices are far from being standardized or regulated. This is a major challenge for the aquaculture industry.

Medicines and chemicals do form a part of the integrated package for fish health management. But their indiscriminate and unregulated use in aquaculture endangers both human health and the environment. Regulations and protocols for the use of medicines and chemicals in aquaculture are therefore essential.

To safeguard fish health and food safety and ensuring sustainable farming, fish farmers must have access to a range of properly authorized medicines and chemicals. The distribution and supply of aquaculture medicines must be carefully regulated. Environmental risk assessment should also be carried out before medicines are authorized.

Globally, there is considerable interest in ensuring that the medicines in aquaculture are used safely and with minimum impact on human health and the environment. Increasing concerns about resistance to antibiotics in human pathogens is also encouraging efforts to ensure that such drugs are not used in fish husbandry.

In India, many antibiotics have been banned from use in aquaculture. A monitoring mechanism is being instituted to ensure that farmers do not use banned antibiotics and other drugs and chemicals. These issues are vital for the industry's sustainability and competitiveness in the domestic and global food markets.

This compendium provides state-of-the-art knowledge on medicines and drugs used in aquaculture. It will serve as a valuable source of reference for aqua farmers, planners, students and scholars working on fin and shellfish health management. I compliment all the authors of the compendium. Special praise is due for Dr I S Bright Singh and Dr Y S Yadava for their effort in editing and compilation.

**Chennai**
30 November 2005

**G Ramanujam**
Chairman
Aquaculture Authority

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# Contents

# Introduction

Aquaculture is a fast-growing food production sector– and a potentially important player in the global food sector. With burgeoning population and growing food requirements, the contributions of aquaculture to the overall fish production are becoming significant.

In 2005, aquaculture accounted for 48 million tonnes of the world fishery production of 142 million tonnes, according to preliminary FAO estimates. This means aquaculture (excluding aquatic plants) made up 34 percent and capture fishery 66 percent of the total global fish production.

Within aquaculture, inland resources provided 29.2 million tonnes (61 %) while the marine sector contributed 18.8 million tonnes (39 %). The FAO time-series data on aquaculture production shows that it rose 80 percent over a period of nine years, from 26.6 million tonnes in 1996 to 48 million tonnes in 2005.

In India too, the share of aquaculture in total fish production has gone up significantly in recent years. In 2004-05, the inland sector contributed 3.44 million tonnes to the total fish production of 6.40 million tonnes. Within the inland sector, about 75-80 percent of the production came from aquaculture (mainly Indian major carps), the balance from capture fisheries. Two decades ago, the share of aquaculture in total inland fish production was a meagre 15-20 percent.

Inevitably, the rapid strides of aquaculture during the last decade have brought many issues to the fore. They relate to food safety, human health and environmental sustainability. The use of drugs and chemicals in aquaculture has raised many public concerns about human health and safety, and about environmental impacts. There is particular concern about the use of antibiotics in aquaculture– which may lead to transfer of drug resistance in pathogenic bacteria from farmed animals to humans. Drug-resistant bacteria are an important concern from a public health perspective– they not only reduce treatment alternatives, they can also decrease the efficacy of treatment on fish farms.

Simultaneously, disease outbreaks and health management of farmed animals have emerged as important limiting factors to aquaculture production and trade. Fish mortalities result in a direct loss of investment in seed, feed, labour, and other input costs– something small and marginal farmers can ill afford to lose. Disease, particularly chronic infections, can have an even larger, though often unnoticed effect on fish production through reduced growth and feed efficiency. Therefore, the problems associated with diseases in farmed fish species, the limited number of drugs available for treatment and prevention of these diseases, and the safety of aquaculture products for human health in general, represent major challenges for the future growth of aquaculture.

Fin and shellfish farmers use a wide range of drugs and chemicals. These comprise legal and illegal drugs, and also the general-purpose chemicals that are not labeled for drug use. Use of drugs and chemicals in aquaculture varies significantly from country to country.

This variation occurs as a consequence of different drug approval requirements and regulatory framework. Example: Japan has 29 individual or combination antibiotics approved for use in aquatic animals, while the United States has five drugs currently legal for use in aquaculture. These include three antibiotics and a combination drug. There are no approved antibiotics for production of crawfish, shrimp, or indeed most other aquaculture species.

In the United States, the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) strictly regulate the use of drugs and chemicals by the aquaculture industry. All drugs legally used in aquaculture must be approved by the FDA's Center for Veterinary Medicine. Standard information on approved drug uses is available in the FDA's 'Green Book' (http://www.fda.gov/cvm/Green_Book/aboutgbook.html).

Such information includes drug ingredients, manufacturer, species, route of delivery, dose form, withdrawal time, tolerance, and uses by species, including dose rates and limitations. The FDA requires a scientific evaluation of a drug's effectiveness and safety for humans and the environment before approval. The EPA requires a scientific evaluation of the safety of chemicals before they can be registered and sold.

While the use of veterinary medicines, biologicals (vaccines) and pharmaceuticals (chemicals) in India is well regulated, there are no such regulations for use of chemicals, drugs and medicines in aquaculture. Because of lack of data and the absence of rules, regulations, jurisdictions, etc., it is becoming increasingly difficult to establish the exact level of drug use and the potential dangers on human health and environment. Further, there are no public sources of aquaculture drug use data and no solid basis to estimate antibiotic drug use.

The volume of antibiotics used in aquaculture is small compared to those in veterinary, industry or in human medicine. However, approved antibiotics in aquaculture are very few. The bottom line is that we must promote aquaculture practices that minimize the risk of antibiotic resistance and ensure that it does not become a public health problem. Continued consumer confidence is critical to the aquaculture industry.

The Food and Agriculture Organization (FAO), the World Health Organization (WHO), and the World Organization for Animal Health (OIE) are working to develop a common approach to deal with the worldwide containment of anti-microbial resistance. Within the international community, there is considerable interest in harmonizing the drug approval process and ensuring that drugs used in aquaculture throughout the world are used safely with no impact on human health or the environment.

In the wake of an influx of a variety of aquaculture drugs, drug formulations and chemicals in the Indian aquaculture sector from within and outside the country, there is a pressing need to regulate the manufacture, import, marketing, prescription and practice of aquaculture medicines to maintain sustainability in aquaculture production.

Indian aquaculture uses a range of chemicals and drugs, which may be categorized as prophylactics, therapeutics and general management chemicals. The distribution and supply of such chemicals and drugs need to be controlled in the right manner. Their authorization

should mean that environmental risk assessment has been carried out to accepted international standards. There should be strict monitoring and enforcement of drug use in aquaculture. These measures could include on-farm visits to review usage before receipt of the product, review of drug usage records on receipt of the product, drug residue testing, impacts of use on human and fish health, etc. Simultaneously, legislation should follow on the safe use of chemicals in aquaculture.

Consumer safety should be addressed by setting up of maximum residue limits (MRLs) derived through toxicological risk assessments. A system should be developed for the environmental risk assessment of chemicals used in aquaculture. It is recommended that the supply and use of fish medicines is uniformly regulated and supported by appropriate codes of best practices. It is also necessary to observe the required withdrawal time before harvest, on the basis of directives from food and hygiene authorities. These issues are very important for the continued competitiveness of the aquaculture sector in the domestic and international food markets.

In view of the above requirements– and the need for a platform to identify different facets of aquaculture medicine in its global perceptive– the Centre for Fish Disease Diagnosis and Management (CFDDM, later renamed as the National Centre for Aquatic Animal Health - NCAAH) conducted a National Workshop on Aquaculture Medicine during 18-20 January 2001 at Kochi.

As an outcome of the Workshop, the Advisory Board (Management) of the CFDDM met on 20 December 2001 and constituted a High-Level Expert Committee on Aquaculture Drug Regulations with experts drawn from National-level Research Institutions and Universities. Dr Y S Yadava, Member Secretary, Aquaculture Authority, Government of India, was nominated as Chairman of the Committee; Dr I S Bright Singh, Coordinator, CFDDM, as its Convenor; and the Advisory Board (Management) as the Steering Committee. The Expert Committee is a standing body with nominated members from fisheries and related Institutions in India.

In the first meeting of the Expert Committee, held at Kochi on 17 January 2002, followed by a three-day Workshop on Aquaculture Drugs from 18 to 20 January 2002, it was decided to bring out a compendium on aquaculture drugs and aquatic animal health management. Subsequently, the Aquaculture Authority at its 26$^{th}$ meeting held on 12$^{th}$ March 2002 endorsed the idea of a compendium.

This compendium is the outcome of the valuable contributions of the High-Level Expert Committee. It provides a review of chemicals generally used in aquaculture, their mode of usage, applications, etc. It covers antimicrobials, anti-parasitic agents, weedicides, general management chemicals, feed additives, hormones, vaccines and immunostimulants. The compendium also covers aspects such as potential delivery systems for aquaculture drugs and nutraceuticals, and hazard analysis and critical control points.

Most importantly, the compendium provides guidelines for regulation of aquaculture drugs in India.

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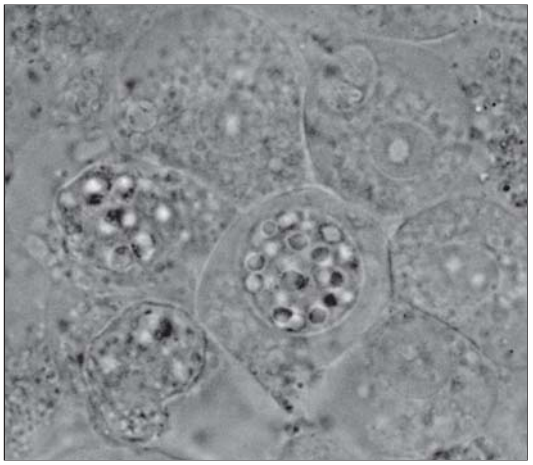


CHAPTER 1

# Antimicrobials in Aquaculture
## (Antifungal, Antibacterial and Antiviral Compounds)*

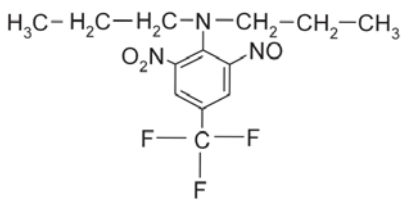
Antimicrobials in aquaculture have become a reality with the expansion of fish farming activities globally. To attain sustainability in production, judicious use of antimicrobials has contributed substantially. To make the applications most effective and environment-friendly, it is essential to know several vital attributes of the compounds handled during disease management. This chapter deals with the most important antifungal, antibacterial and antiviral compounds used in aquaculture.

## A. Antifungal compounds

### 1. Trifluralin

#### *1. Chemical name and structure*

The chemical name: a,a,a-Trifluoro-2,6-dinitro-N,N-dipropyl-p- toluidine. Emperical formula: $C_{13}H_{16}F_3N_3O_4$. Trade names include, Flurene SE, Treflan, Tri-4, Trust, M.T.F., Trifluralin 600, Elancolan, Su Seguro Carpidor, Trefanocide, Treficon, Trim, L-36352, Crisalin, TR-10, Triflurex and Ipersan.




*The structure of trifluralin*

#### *2. Target organism*

Trifluralin is commonly used as a prophylactic chemical against fungal infections such as larval mycosis in shrimp caused by *Lagenidium callinectes*, *Haliphthoros,* etc.

#### *3. Mode of action*

Inhibits cell wall synthesis of fungi.

#### *4. Mode of application*

Dilute in water and apply to the rearing water in the required quantity.

#### *5. Shelf life and storage*

At refrigerated conditions it remains stable for more than two years.

#### *6. Recommended dosage*

Dosage of 0.2 to 1.0 ppm level with no inhibitory effect on hatching rate of eggs of *Penaeus monodon*. However, survival rate of hatched nauplii subsequent to treatment will be significantly reduced in most cases. Treflan at 0.01 ppm is recommended to treat *Lagenidium* infections.

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***7. Metabolism of the drug in animal body, retentivity and mode of excretion***

The drug is not known to be absorbed by animal body.

***8. Residues in host species***

No residual effect in host species is recorded.

***9. Host species safety***

Application in the recommended dosage is safe for the host species.

***10. Environmental safety***

Exposure of the non-target species such as *Teraselmis cheuii*, a phytoplankton used as food for peneaid larvae, to 0.1 ppm trifluralin delays its growth and reduces protein content. The drug gets degraded in soil water systems.

***11. Operator safety***

Handle the drug with the precautions given for a bio-active chemical component.

**2. Griseofulvin**

Also known as gris- PEE, Erisactin, Grifulvin V and Fulucin. It is derived from the fungus *Penicillium griseofulvum*. Poorly water soluble, and as antifungal agent effective against common dermatophytes *Microsporum*, *Epidermophytum* and *Trichophyton*. Griseofulvin disrupts mitotic spindle structure leading to metaphase arrest.

**3. Azole drugs**

***Econazole nitrate***

Econazole nitrate is an azole derivative. With respect to antifungal activity, this decreases conversion of 14-alpha-methylsterols to ergosterol, an important membrane component in fungi. Failure of ergosterol synthesis causes altered membrane permeability leading to loss of ability to maintain a normal intracellular environment. The target organisms are *Lagenidium* and *Haliphthoros* in shrimp larvae. Residues up to 1 ppm level show no inhibitory effect on hatching rate of eggs of *P. monodon*, but survival rate of hatched nauplii is significantly reduced in most treatments.

**4. 2,4 – D**

***1. Chemical name and structure***

2,4 – Dichlorophenoxy acetic acid is a chlorinated phenoxy compound.

***2. Target organism***

*Legenidium* and *Haliphthoros* in shrimp larvae. Used to control many types of broad leaf weeds in aquaculture and in pastures and range land applications, forest management, homes and gardens and for the control of aquatic vegetation.

***3. Mode of action***

Functions as a systemic herbicide.

4. ***Mode of application***

   Dissolve in water and apply as per requirement in the rearing water.

5. ***Shelf life and storage***

   Stays for more than a year, provided it is kept in a cool dry place.

6. ***Recommended dosage***

   Dosages of 0.2 to 1.0 ppm in shrimp larval rearing systems are recomended. As weedicide, higher concentrations ranging from 5 to 10 ppm are required.

7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

   Even though absorbed into animal body, its metabolism is not worked out.

8. ***Residues in host species***

   Residues in host species up to 10 ppm are reported.

9. ***Host species safety***

   Exhibits no inhibitory effect on hatching rate of eggs of *P. monodon*, but survival rate of hatched nauplii is significantly reduced in most treatments.

10. ***Environmental safety***

    In soil water systems the chemical gets degraded.

11. ***Operator safety***

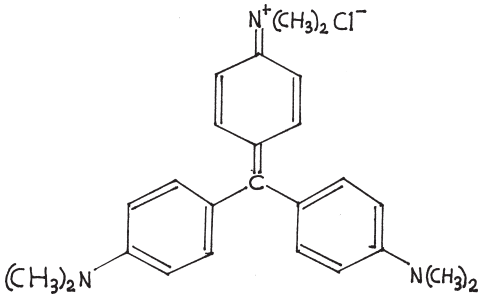
    Handle the drug with precautions given for a bio-active chemical component.

**5. Crystal violet**

Chemical name and structure*:* $C_{25}H_{30}N_3Cl$, Hexamethylpararosaniline chloride. The target organisms include *Lagenidium* and *Haliphthoros*. It has high bactericidal activity also.

***Residues in host species***

Up to one ppm level, no inhibitory effect is shown on hatching rate of eggs of *P. monodon* but survival rate of hatched nauplii is significantly reduced in most treatments.




*Crystal violet*

**6. Malachite green**

1. ***Chemical name and structure***

   Malachite green (p,p-benzylidenebis-N, N-dimethyl aniline).

2. ***Target organism***

   *Saprolegnia* and external protozoans.

3. ***Mode of action***

   Believed to bind strongly with internal cytoplasmic structures of parasites and interfere with normal metobolism. It is a respiratory poison.

4. ***Mode of application***

   Bath treatment

5. ***Shelf life and storage***

   Stays indefinetly, provided it is kept in a cool dry place.

6. ***Recommended dosage***

   Concentrations of malachite green ranging from 0.1 to 0.5 ppm in a variety of application methods have been recommended. The concentration used to treat fungi (water mould) in fish eggs is usually 5 ppm for 15 minutes to one hour. A mixture of formalin and malachite green in a ratio of 25:0.1 ppm appears to be very effective to treat "Ich" in both aquarium and food fishes. Fungal infections in milkfish are controlled using baths of 10-ppm malachite green for an undisclosed period. Malachite green at 1 % is swabbed directly on dermal lesions and fins to control secondary fungal infections. Juvenile fishes with suspected fungal infections are dipped in 100 ppm malachite green for a few seconds. In *P. monodon* hatcheries in the Philippines, every other day from Mysis 1 to post-larvae, long bath at a dosage of 0.003-0.015 ppm is found to be applied.

7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

   Metabolism of the drug in animal body is not known but malachite green has carcinogenic properties and causes chromosome defects and leukopenia in treated trout.

8. ***Residues in host species***

   One ppm bath of malachite green (topical fungicide/parasiticide) for an hour can produce a blood serum level in fish of up to 14 mg/kg at temperate water temperatures. Its elimination (excretion) from fish tissues is extremely slow. More than 200 days at $10^{o}$ C are required for the residues to be eliminated.

9. ***Host species safety***

   Malachite green has carcinogenic properties and causes chromosome defects and leukopenia in treated trout.

10. ***Environmental safety***

    Malachite green gets degraded very slowly in water sediment systems.

11. ***Operator safety***

    Since it has carcinogenic properties, handling should be with all precautions of dealing with such category of compounds.

## B. Antibacterials

### a. Antibiotics *(Warning: Antibiotics are banned for use in aquaculture in India).*

### 1. Tetracyclines

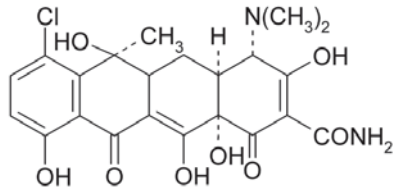
#### *1. Chemical name and structure*

Tetracyclines

Natural tetracyclines: oxytetracyclines (OTC) and Chloretetracyclines (CTC)

Semi-synthetic: tetracyclines and doxycyclines

These are yellow crystalline compounds with low octanol/water partitioning coefficients and varying solubilities in water; soluble in both acids and alkalies. They are chelating agents and form complexes with divalent cations, which are microbiologically inert. Unstable in alkaline solution. Complex with organic materials, especially proteins and clay.

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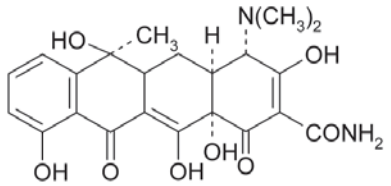


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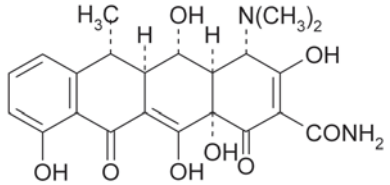



(c)

*(a) Chlortetracycline*
*(b) Oxytetracycline*
*(c) Doxycycline*

#### *2. Target organism*

Broad-spectrum antibiotics, especially OTC have been widely used in aquaculture as they are available in most markets and comparatively less expensive also. Because of the long use, several bacterial pathogens have become resistant to it. The spectrum of activity includes *Vibrios, Aeromonas*, *Yersenia*, *Flavobacterium*, *Flexibacter*, *Streptococcus,* etc.

#### *3. Mode of action*

The tetracyclines, which are amongst the most frequently used of all broad spectrum antibiotics, are octahydro-naphthacenes, isolated as chlortetracycline in 1947. All tetracyclines depend on a preferential accumulation in bacteria for their selectivity, forming a liposoluble complex with magnesium in the bacterial plasma membrane. In the cytoplasm, tetracyclines become bound to the 30S ribosome unit and inhibit protein synthesis. Tetracyclines are chelating agents; they may be inactivated by calcium and magnesium ions. Thus, water hardness may also have relevance to the efficient use of tetracyclines

in fish. Apart from antibacterial action, OTC is used as a growth promoter in some cyprinoid species by feeding orally at 50 mg/kg/day.

***4. Mode of application***

Injection for larger animals, which are not feeding, oral administration through diet and immersion. Mixing OTC with diet leads to a reduction in intake by 61 %, and therefore feed medication rates should be 65 % above the required rates. Bioavailability studies conducted in rainbow trout and salmon indicated 2.6 to 8.6 % of the antibiotic administered with a total absorption of 9 % at 14$^{o}$ C. In comparison with other antibacterial agents, the oral bioavailability of OTC is very low in all fish species. Temperature has great significance in the rate and extent of absorption on oral administration. Data on (T*max*) time of peak absorption and (C*max*) maximum concentration achieved are available only for a limited number of species. Higher temperatures may give a low T*max* but will lead to low C*max* due to rapid excretion. For fishes, which are not feeding and too large for immersion treatment, injection is the only option. Intraperitoneal route is the best choice as it causes less necrosis.

***5. Shelf life and storage***

Stable as dehydrated powder form at 4$^{o}$ C, but unstable in solution, particularly in alkaline solution. Injections are, therefore, often formulated as hydrochlorides in non-aqueous carriers.

***6. Recommended dosage***

One injection of 20 mg/kg body weight for brood fish would be sufficient as the drug is relatively persistent in the fish body. On administering through diet, 75 mg/kg body weight/day in the case of marine fishes or less in the case of freshwater fishes over a period of 10 days would be required.

For immersion treatment, the required concentration ranges from 5 to 120 ppm depending on the hardness of water. OTC complex with $Ca^{++}$ and $Mg^{++}$ ions get inactivated and depending on the hardness of water the dose has to be altered. OTC cannot be used therapeutically but may be of use in preventing transmission of water borne bacterial diseases.

LC values are 840 ppm for one hour immersion treatment and 55 mg/kg body weight by injection once weekly for 4 weeks. A dose of about 200mg/kg body weight causes slower growth rate.

Both oxytetracycline and chlortetracycline were examined for their potential as fish chemotherapeutants shortly after they became available for medical use. In natural outbreaks of ulcer disease of brook trout caused by *Haemophilus piscium,* it was found that oxytetracycline supplied orally at 75 mg/kg body weight of brook trout fingerlings per day had a marked beneficial effect, provided the treatment commenced well before the infection was too far advanced. Oxytetracycline has always proved

superior in performance to chlortetracycline, which showed no improved survival compared to controls.

Other pathogens against which oxytetracycline has been tested include *Chondrococus columnaris,* the causative agent of columnaris disease, which was inhibited by 10 µg of oxytetracycline *in vitro. Aeromonas liquifaciens* can be controlled in golden shiners by an 8-day regimen of 55 mg/kg body weight per day and *Pseudomonas* in white catfish where abnormal mortalities ceased after 4 days treatment with oxytetracycline at 55 mg/kg per day. Excellent results against fin rot were reported for oxytetracycline or oxytetracycline-streptomycin medicated baths. Control of streptococcal infections in golden shiners has also been reported by applying oxytetracycline as a medicated bath at 5-120 ppm.

***7. Metabolism of the drug in animal body, retentivity and mode of excretion***

Highest concentration of OTC is found in liver tissue besides its therapeutic concentration in skin, mucus and vertebrae. OTC in blood is bound to plasma proteins to the extent of 52 - 55%. The half life of OTC in rainbow trout, especially in serum, muscle and liver is temperature dependent within a temperature profile of $5^0$ C to $16^o$ C. Accordingly, the half life in serum ranges from 8.9 ± 2.3 to 4.8 ± 1.3, in muscle 8.8 ± 3.9 to 5.1 ± 0.5 and in liver 9.5 ± 2.1 to 4.7 ± 0.9 days. Based on this data, the withdrawal period is calculated as 92, 45 and 37 days in temperature regimes of $5^0$C, $10^0$ C and $16^0$ C respectively. Data from the tropics are not available and considering the pattern described earlier the withdrawal period can be again shortened to 15 days as the average temperature ranges from $25^0$ C to $30^0$ C and OTC excretion is a linear function of temperature.

***8. Residues in host species***

Tetracyclines are deposited in bones of fishes as much as in higher vertebrates. The limit of detection of OTC is 0.05 ppm in muscle, kidney and blood and 0.01 ppm in skin.

***9. Host species safety***

Although OTC is primarily used for its antimicrobial action, it has a pharmacodynamic effect on fish as an immunosuppressor. By either route of administration, the drug produces 20 percent fall in total serum protein. A profound drop in immunoglobulin is experienced with a single injection a day before the administration of antigen, but no drop is seen if the OTC and antigen are given simultaneously. OTC interferes with normal monocyte activity, which results in a low response to a primary antigenic stimulus, but that with the secondary stimulus, the antigen is trapped more efficiently. The mode of action of OTC on monocytes is probably inhibition of mitosis. No difference in granulocytes or platelet count was observed, but the total number of lymphocytes and monocytes is significantly reduced by medication. OTC injection leads to a reduction in the erythrocytic count.

***10. Environmental safety***

The bioavailability of oral OTC is low. A large part of the drug used in aquaculture enters the environment through food and faeces. A portion is subjected to ingestion by wildlife. OTC residue has been recorded in the musculature of wild fish for one week after the application. Further, biologically significant concentration of OTC gets into the organic deposits in sediment from farms where it is persistent and it is found to increase in sediments at depths greater than 2 cm for 19 days after the end of a course of therapy. When OTC diffuses into mud, it probably has very little biological activity. It binds to humic acid, peat and clay forming antimicrobially inactive complexes, as the divalent cations and organic sediments reduce the activity 100 fold. Two environmental hazards are the killing of beneficial flora, especially the bacteria which oxidize sulphides to sulphates, and the induction of transmissible resistance. In the light of profound inactivation of OTC by both sediment and divalent ions, the concentration required for efficiency has been determined as high as 100-200 ppm. Half-life of OTC in sediment depends on the physical and chemical characteristics of the sediment rather than any inherent property of OTC. It can get depurated into water by dissolution. The concentration, which it can attain in water column by this means, is negligible.

***11. Operator safety***

No incident of any risks involved in handling OTC has been reported. But it should be handled with all precautions and care required for handling of drugs.

**2. ß-lactam antibiotics**

**Ampicillin**

***1. Chemical name***

b-(2-amino-2-phenylacetamido) penicillic acid.

***2. Target organism***

Effective on a wide variety of Gram negative and Gram positive bacteria. Several resistant strains of *Edwardsiella tarda, Vibrio parahaemolyticus, Vibrio alginolyticus, Aeromonas liquefaciens, Aeromonas hydrophila* and many strains of *Pseudomonas* differentially sensitive have been isolated.

***3. Mode of action***

Interferes with the final stage of peptedogycan biosynthesis, inhibits the transpeptidase reaction, namely the cross linking of the two linear polymers.

***4. Mode of application***

Orally as coating on feed.

5. ***Shelf life and storage***

Remains stable for one to two months if maintained tightly closed in cool dry place.

6. ***Recommended dosage***

The dosage of ampicillin for *Pasturella piscicida* is 0.04 µg/ml. For other organisms not specifically recomended.

7. ***Metabolism of the drugs in animal body, retentivity and mode of excretion***

No information is available on the metabolism of the drug in fish and prawns.

8. ***Residues in host species***

Residue level of 0.03 ppm in fish has been detected.

9. ***Host species safety***

No deleterious effect on finfish and shellfish at the recommended concentrations has been reported.

10. ***Environmental safety***

Generally, the drug gets degraded in soil water system easily, but disruption of microbial equilibrium at least for a short while is expected.

11. ***Operator safety***

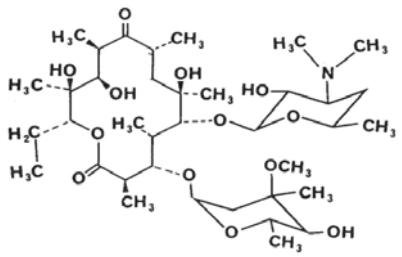
Generally safe, but should not be brought in contact with eye and skin.

**3. Macrolides**

**Erythromycin**

1. ***Chemical name and structure***

14-ethyl-7,12,13-trihydroxy-4-([2R, 4R 5S, 6S)5-hydroxy-4-methoxy-4, 6di methyloxan 2-yl]oxy)



2. ***Target organism***

Targeted for Gram-positive bacteria, principally bacterial kidney disease (BKD) and streptococcosis in fishes.

3. ***Mode of action***

Erythromycin inhibits protein synthesis as a result of binding on 50S sub-unit ribosome; the steps of transpeptidation and translocation in protein synthesis are blocked.

4. ***Mode of application***

The antibiotic is either coated on diet or given as injection.

5. ***Shelf life and storage***

Remains stable for two to three months if maintained tightly closed in a cool dry place.

***6. Recommended dosage***

For the chronic disease BKD (caused by the Gram-positive bacterium *Renibacterium salmoninarum),* erythromycin was found as the most effective antibiotic at a dosage of 100 mg/kg/day for 21 days.

***7. Metabolism of the drug in animal body, retentivity and mode of excretion***

No information is available on the metabolism in fish and prawn.

***8. Residues in host species***

Information on its residual effect in fish and prawns is not available.

***9. Host species safety***

Erythromycin at 10 mg/kg/day can cause lesions in the kidney of fishes, especially to the first proximal segment of nephron, where within 24h of treatment the cell lining is found with extensively vacuolated cytoplasm.

***10. Environmental safety***

Erythromycin gets degraded fast in soil water system.

***11. Operator safety***

This compound should not be brought in contact with eye and skin.

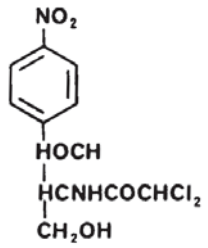
**4. Phenicols**

**Chloramphenicol**

***1. Chemical name and structure***

2,2-dichloro-N-[1,3-dihydroxy-1-(4-nitrophenyl)propane-2-yil acetamide]

***2. Target organism***




Chloramphenicol is effective against *Aeromonas hydrophila* and *A. liquifaciens.* It is also highly effective in both *in vitro* and *in vivo* against *Haemophilus piscium* and *A. salmonicida,* particularly in eliminating latent infections.

***3. Mode of action***

Chloramphenicol, an antibiotic produced by *Streptomyces venezuelae,* possesses a wide antimicrobial spectrum, but is largely bacteriostatic rather than bactericidal. In bacteria it acts primarily on the 50S ribosomal sub-unit appearing to uncouple ribosomal translocation from peptide bond synthesis. Soon after its introduction into medicine, resistance was encountered and its use became associated with fatal blood dyscrasias, which may also be due to inhibition of protein synthesis.

4. ***Mode of application (including recommended preparations required for applying in aquatic system)***

Bath treatment, injection and/or oral administration, mostly as medicated feed. However, FDA regulations ban chloramphenicol from use in animals intended for food production. The Canadian Health Protection Branch, the European Union and Japan apply the same measure. Hence, mode of formulation for aquaculture has not been developed.

5. ***Shelf life and storage***

Remains stable for two to three months if maintained tightly closed in cool dry place.

6. ***Recommended dosage***

Dose rates of 50-100 mg/kg fish/day for 5-10 days have been recommended or as routine prophylactic intraperitoneal injection as a single dose at 12-mg/kg-body weight.

7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

No information is available on the metabolism in fish and prawns.

8. ***Residues in host species***

Daily treatment of chloramphenicol has proven to be cumulative in muscle.

9. ***Host species safety***

The adverse effect of chloramphenicol on fish is seen as major disturbances in its erythropoiesis after a single application.

10. ***Environmental safety***

The drug gets degraded in the environment quickly, but before that it may alter the microbial equilibrium, at least temporarily.

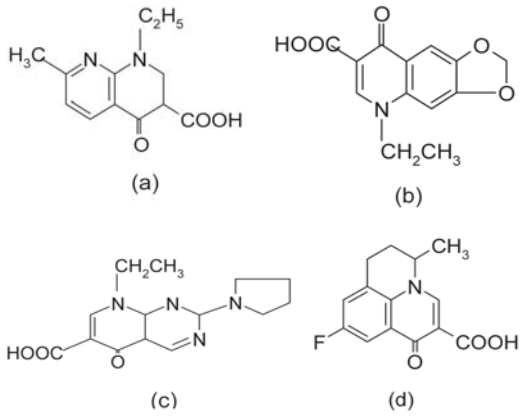
11. ***Operator safety***

This compound should not be brought in contact with eye and skin.

**5. 4-Quinolones**

1. ***Chemical name and structure***

The 4-quinolones are synthetic systemic antibacterial agents, which include nalidixic acid, piromidic acid, oxolinic acid and flumequine.




*4 – Quinolone antimicrobial agents used in aquaculture (a) Nalidixic acid; (b) Oxolinic acid; (c) Piromidic acid; (d) Flumequine.*

**2. *Target organism***

The antimicrobial spectrum of quinolones is largely limited to Gram-negative bacteria which, however, cause some of the most serious fish diseases. Oxolinic acid and flumequine are used for the control of furunculosis and enteric red mouth disease in farmed fish. Oxolinic acid is effective against *A. salmonicida, A. liquifaciens, V. anguillarum* and *Chondrococcus columnaris*.

**3. *Mode of action***

4-quinolones interfere with the action of bacterial DNA gyrase preventing the completion of the negative supercoiling of bacterial chromosome. While sensitive bacterial cells lose viability from exposure to 4-quinolones, abnormal cell elongation has also been reported.

**4. *Mode of application***

4-quinolone antimicrobials are characterized by extremely low toxicity, high efficacy at low dose and rapid absorption, both by oral and bath techniques.

**5. *Shelf life and storage***

Remains stable for two to three months if kept tightly closed in a cool dry place.

**6. *Recommended dosage***

The recommended dosage of oxolinic acid for *A. salmonicida, A. liquifaciens, V. anguillarum* and *C. columnaris* is 0.02 to 0.09µg/ml. Oxolinic acid and nalidixic acid are effective at 2.0 ppm in bath treatment of fishes infected by *Pseudomonas anguilliseptica* and oxolinic acid and piromidic acid are effective at 5.0µg/kg. A new 4-quinolone antimicrobial (flumequine) is effective against furunculosis in fishes with a dose rate of 12mg/kg body weight/day for practical fish farm usage.

**7. *Metabolism of the drugs in animal body, retentivity and mode of excretion***

*Oxolinic acid:* It is recommended that withdrawal periods for oxolinic acid be 14-21 days for fishes.

*Piromidic acid:* The hydroxylation product of piromidic acid (α- and β-hydroxy-piromidic acids) shows greater antimicrobial activity than the parent compound. Both piromidic acid and its bioactive metabolites disappear entirely from fishes by 48h.

**8. *Residues in host species***

Given sufficent time, no residue will be left in fish/prawn body. Information is not available on its residual effect on non-targeted organisms.

**9. *Host species safety***

Oral administration to fishes with a single dose of oxolinic acid of 50µg/ml shows partial mortality and at 100µg/ml it is lethal to all fishes within 48 hours. Both oxolinic acid and nalidixic acid produce a slight loss of appetite and darkening of the skin.

Erythrocytes become markedly macrocytic and the liver shows slight fat reduction with hepatocyte atrophy.

***10. Environmental safety***

It gets degraded fastly in soil water system.

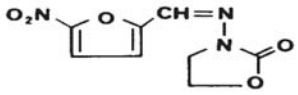
***11. Operator safety***

The compound should not be brought in contact with eye and skin.

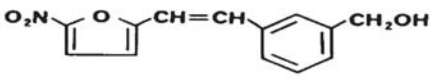
**6. Nitrofurans**

***1. Chemical name and structure***

The nitrofurans are nitroheterocyclins. The defining structural component is a furan ring with nitro group.




*(a) Furazolidone*




(*b) Nifurpirinol*

***2. Target organism***

Furazolidone is effective against furunculosis in fishes. A new nitrofuran, initially coded P-1738 and known as nifurpirinol (Furanace) is effective against a wide range of Gram-negative and Gram-positive bacteria. It is also effective against *Cytophaga columnaris, Saprolegnia parasitica* and *Trichomonas vaginalis*.

***3. Mode of action***

The mode of antibacterial action of these compounds involves blockage of the initiation of translation of inducible enzymes and to the inhibition of enzymatic repair of nitrofuran-induced lesions in DNA. This antibacterial mode of action is intimately related at the molecular level to a mutagenic activity of nitroheterocyclic compounds.

***4. Mode of application***

Nifurpirinol is effective both by bath and by oral application methods at low dose levels.

***5. Shelf life and storage***

Remains stable for two to three months, if maintained tightly closed in a cool dry place.

***6. Recommended dosage***

Furazolidone at a dose 100 mg/kg for 14 days is effective against furunculosis in fishes. Nifurpirinol is effective by single bath application against columnaris in fishes at 0.5 ppm for Ih at 21°C and by three daily baths of 1.0 ppm to control other diseases

caused by members of Cytophagaceae. In salt water ponds, oral therapy with nifurpirinol at 50 and 75 mg/kg for 14 days at 20° C appeared to control vibriosis in fishes. Sodium nifurstyrenate at a dose of 30-100 mg/kg body weight is effective against pseudotuberculosis caused by *Pasturella piscicida.*

***7. Metabolism of the drug in animal body, retentivity and mode of excretion***

No information is available on the metabolism in fish and prawn. The drug gets excreted within 72 hours.

***8. Residues in host species***

Informations on its residual effect in the host species is not available.

***9. Host species safety***

No toxic effect is noted in 30 minutes baths of 30µg/ml or in the host species fed at 200mg/kg for three days. Oral application of nifurpirinol (up to 100 mg/kg) to control furunculosis in fishes has failed due to palatability problems. Toxicity of nifurpirinol in fishes exposed at 0.5 ppm continuously for up to 14 days includes skin lesions, which sometimes develop into skin and muscle erosion.

***10. Environmental safety***

The drug gets degraded in the environment quickly. But changes in the microbial equilibrium may be caused at least temporarily.

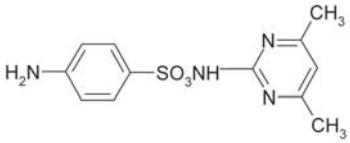
***11. Operator safety***

The compound should not be brought in contact with eye and skin.

**7. Sulphonomides**

***1. Chemical name and structure***

*(a) Sulphadimidine (sulphamethazine), (b) sulphanilamide, (c) sulphamerazine, (d) sulphadiazine, (e) sulphathiazole, (f) sulphasoxazole, (g) trimethoprim*

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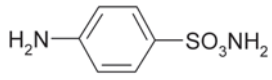


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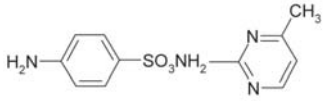


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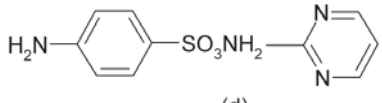


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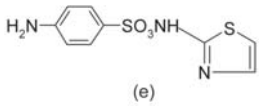

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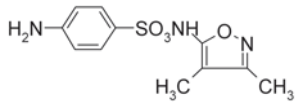

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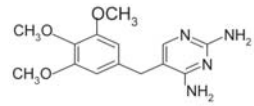


(g)

***2. Target organism***

The sulphonamides are effective against Gram-negative bacteria such as *Aeromonas salmonicida,* the aetiologic agent responsible for furunculosis. It is also used for the control of enteric redmouth disease in fishes, which is caused by the Gram-negative bacterium, *Yersinia ruckeri.*

***3. Mode of action***

Sulphonamides are antagonists of dihydrofolic acid biosynthesis, competing with the natural metabolite *p-aminobenzoic* acid. Susceptible bacteria are unable to absorb di-hydrofolic acid. Potentiated sulphonamides react by means of a sequential blocking of dihydrofolate reductase by 2,4-diaminopyrimidines (*e.g.* trimethoprim).

***4. Mode of application***

Oral and as bath treatment.

***5. Shelf life and storage***

Remains stable for two to three months if maintained tightly closed in a cool dry place.

***6. Recommended dosage***

Sulphamerazine supplied orally to fishes at a dose of 20 mg/kg fish body weight/day is found to be effective in controlling furunculosis. For bacterial kidney disease, multiple sulphonamide therapy of which sulphamerazine, sulphaguanidine and sulphadiazine, either alone or in various combinations at 250 mg/kg bodyweight/day, is highly effective

***7. Metabolism of the drugs in animal body, retentivity and mode of excretion***

The sulphonamides differ in their absorption and excretion characteristics, which largely determine their performance *in vivo*.

***8. Residues in host species***

An 80-day withdrawal period of trimethoprim is advisable in winter temperatures for fishes. The sulphamerazine residues approach zero in fish muscles at 20 to 40 days after a withdrawal period. In fishes maximum sulphamerazine concentrations are detected in the liver, where significant proportions are acetylated with lesser amounts in kidney, blood and muscle. The acetylated metabolite of sulphamerazine does not possess significant antibacterial activity.

***9. Host species safety***

Published information on toxic effects of sulphonamides on fish at therapeutic levels is fairly limited. Although fishes are capable of utilising available dihydrofolic acid, inhibition of dihydrofolic acid synthesis may have a marked effect on the growth of young actively growing fishes. Decreased growth in sulphonamide treated fish has been seen. Sterility, kidney damage and mortalities are also reported in fish treated with sulphamethazine. Moribund fish exhibit swollen stomach, marked constrictions just anterior to the gastric caeca, and sulphonamide crystals are also seen in the kidney. Sulphadimethoxine, although superior to sulphadimidine, is found to be less readily accepted by fish and is less active than sulphasoxazole.

***10. Environmental safety***

Even though the drug gets degraded in the environment, it can cause temporary changes in the microbial equilibrium.

***11. Operator safety***

The compound should not be brought in contact with eye and skin.

**b. Antibacterials other than Antibiotics**

**1. Iodine**

***1. Chemical name and structure***

Iodine ($I_2$) is available as Polyvinyl pyrrolidone iodine (PVPI) /Iodophor compounds. These preparations are complexes of Iodine with a solubilizing agent or carrier that liberates free iodine into solution slowly.

***2. Target organism***

They can prevent and control diseases caused by *Aeromonas*, *Pseudomonas*, *Vibrio*, and fungi and several viruses. These compounds are particularly used to treat eggs and larvae, and to disinfect equipment. Lethal to viruses, which are killed within 15 minutes in a 50 ppm solution.

***3. Mode of action***

Iodine is an oxidizing agent, which can invariably oxidize and inactivate proteins with Sulfhydril groups (-SH group). The action involves halogenation of tyrosine units of enzymes and other cellular proteins requiring tyrosine for activation.

***4. Mode of application***

Iodine is available as complexes of Polyvinyl pyrrolidone iodine, which can release iodine slowly in water. This preparation is diluted in water and sprayed as per requirement.

5. ***Shelf life and storage***

   The iodine compounds are to be maintained in air tight dark bottles in a cool dry place. In such situations it can remain stable for long periods.

6. ***Recommended dosage***

   Iodine at 0.02-0.06 ppm, 0.1-0.45 ppm, and 0.3-0.6 ppm is applied on larvae, juvenile and adult fishes respectively as a long-period bath. Shrimp eggs and nauplii are dipped in iodine solution for 30 seconds at concentrations of 200 ppm and 50 ppm respectively, to avoid the introduction of Monodon Baculovirus (MBV) from broodstock and other sources.

7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

   Iodine is not consumed by animal body.

8. ***Residues in host species***

   No residual effect is reported in host species.

9. ***Host species safety***

   Iodine is toxic if added in higher concentration than what is recomended.

10. ***Environmental safety***

    Environmental risk is associated with the disposal of the compounds to soil water system.

11. ***Operator safety***

    No hazardous effect is reported.

**2. Chlorine**

(Refer Chapter 4)

**3. Formalin**

1. ***Chemical name***

   Formaldehyde dissolved in water.

2. ***Functional component***

   Formalin is a generic term, which describes a solution of 37 % formaldehyde gas dissolved.

3. ***Target organism***

   Target on *Trichodina* and *Myxolobus*. Not effective on fishes heavily infested with *Prosthodiplostomum*. At 1 %, it is found to be effective in killing the spores of the histozoic myxosporidian, *Myxobolus vanivilasae* infecting *Cirrhinus mrigala*. Formalin effectively kills parasites on gills, skin and fins. It is not the preferred treatment for external bacterial or fungal infections. In addition, high concentrations of formalin are used to control fungi on fish eggs. Formalin is not effective against internal infections of any type.

4. ***Mode of action***

Acts as a disinfectant, antiseptic and astringent.

5. ***Mode of application (including recommended preparations required for applying in aquatic system)***

For application, dilution is necessary in order to ensure that therapeutic dosages may be safely discharged to receiving water. In most current application, this dilution will occur before discharge by mixing formalin-containing effluent with untreated waste streams from other locations on the farm. Formalin is used as a bath treatment to control external parasitic infections of fish.

6. ***Recommended dosage***

Treatment concentrations are typically 15 to 250 mg/L for treatment of fish and crustaceans and up to 200 mg/L for control of fungi on eggs.

7. ***Metabolism of the drug in animal body, retentively and mode of excretion***

The concentration at which the chemical is applied does not lead to its consumption in the host body.

8. ***Host species safety***

(i) Formalin chemically removes oxygen from the aquatic environment. Each 5 mg/L of formalin applied removes 1 mg/L of dissolved oxygen. This is one reason why use of formalin in ponds is discouraged.

(ii) Formalin is an algicide. When applied to pond water, formalin kills a portion of the algae present, thereby reducing the ability of the algae to produce oxygen through photosynthesis. Further decrease of oxygen in the pond can result as the dead algae decompose.

(iii) Formalin should be stored in an area where it is protected from extremes of heat and cold. Never use formalin when storage temperatures fall below $40^{o}$ F ($5^{o}$ C).

9. ***Environmental safety***

Water quality parameters such as DO, $CO_2$, pH, total ammonia and nitrite are influenced when used at recommended rates as it is toxic to many of the aquatic plants, especially phytoplankton and algae. Formalin is toxic to aquatic life at low concentrations with 96 hrs LD 50 values ranging from 1 to 1000 mg/L depending on species. Formalin toxicity is increased at high water temperatures. If water temperature exceeds $70^{o}$ F ($21^{o}$ C), the concentration used should be reduced.

10. ***Operator Safety***

Formalin is a potential carcinogen and should be handled very carefully to avoid skin contact, eye irritation and inhalation. Formaldehyde is a noxious gas. Formalin must be kept in a sealed container in well-ventilated area. Exposure to fumes will

result in irritation to eyes and respiratory surface. Some people develop sensitivity to formalin over a period of time, which involves repeated handling of the chemical. They should avoid handling the chemical.

**4. Acriflavine**

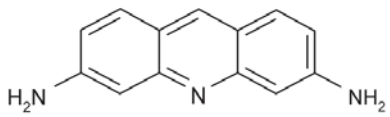
***1. Chemical name and structure***

3,6 - Diamino-10-methylacridin-10-ium chloride.

***2. Target organism***

Targeted at *Costia* spp., *Chilodonella* spp., *Trichodina* spp., *Flexibacter* spp. which cause fin and skin turbidity or cloudiness. This chemical is used to treat fish eggs and aquarium fishes for infection by bacteria and external protozoans.

Acriflavine




$C_{14}H_{14}C_1N_3$

***3. Mode of action***

Binds with nucleic acids of the target organisms.

***4. Mode of application***

Dissolved in water and sprayed over water column of hatcheries and grow out systems.

***5. Shelf life and storage***

Remains stable for two to three months if maintained tightly closed in cool dry place.

***6. Recommended dosage***

The dose of 100 ppm for dipping eggs for 3-5 seconds and 25 ppm for prolonged treatment of *F. columnaris* in sea bass. For walking catfish, only 5 ppm is recommended. For aquarium fish, 5-10 ppm bath for 24 hours is ideal.

***7. Metabolism of the drug in animal body, retentivity and mode of excretion***

No information is available on its metabolism in the animal body.

***8. Residues in host species***

No information is available on its residual effect on host species.

***9. Host species safety***

Since the compound can bind with nucleic acid, higher concentrations may lead to tissue damage.

***10. Environmental safety***

Acriflavin dyes intensely the utensils and aquaculture implements. On using in aquarium in excess it can severely damage the aquarium plants.

***11. Operator safety***

This compound should not be brought in contact with eye and skin.

## 5. Potassium Permanganate

### *1. Chemical name and structure*

Potassium Permanganate ($KMnO_4$). It is a salt consisting of $K^+$ and $MnO_4^-$ ions.

### *2. Target organism*

Not specifically on any organism, but generally on bacteria and fungi.

### *3. Mode of action*

Potassium permanganate ($KMnO_4$) is an oxidizing agent that has been used for many years in aquaculture. It is also used in water conditioning systems and in the plumbing industry. As an oxidizer, it is able to chemically "burn up" organic material. Because the chemical cannot distinguish between desirable and undesirable organic matter, it is up to the user to use the chemical in a manner that results in maximum benefit and minimum harm to treated fish.

When potassium permanganate is active (in its unionized form), treated water turns a pinkish-purple colour. As the chemical is 'deactivated' (by oxidizing organic material), the water colour changes to yellow or muddy brown. This colour change is an important tool when monitoring chemical treatment. As with many chemicals used in water, potassium permanganate is harmful to plants and invertebrates.

### *4. Mode of application*

Dissolve in water and apply as required.

### *5. Shelf life and storage*

As crystals it remains stable indefinitely on maintaining in a cool dry place.

### *6. Recommended dosage*

For most fish species, potassium permanganate can be administered at a concentration of 2 mg/L as long-term bath (4-hour minimum) in fresh water or salt water systems. Potassium permanganate is also reasonably safe to use in recirculating systems and has minimal impact on biofilters when used at 2 mg/L. Treated water should retain the purple coloration for at least 4 hours. Some fishes, including certain lake Malawi cichlids are sensitive to potassium permanganate and lower concentrations (1 mg/L) may be safer. The user can determine species sensitivity by observing the behavior of the fish during treatment. This is especially important when treating a species for the first time. If fish reacts adversely, immediate action (such as diluting the chemical with fresh water) can be taken. Because organic matter deactivates potassium permanganate, it may be necessary to increase the amount by adding to the pond or other systems where organic material has been allowed to accumulate. A safe way of accomplishing this is to add potassium permanganate to the system in 2 mg/L increments. If water colour changes from purple to brown in less than 4 hours from the start of first treatment, an additional 2 mg/L should be added. If a total application of 6 mg/L potassium permanganate does not result in maintenance

of purple color for at least four hours, it indicates that the system contains large quantity of organic matter and ought to be cleaned. Most of the organisms that are treated with potassium permanganate thrive in an organically rich environment; and, therefore, improved sanitation can have a tremendous impact on treatment efficacy.

Potassium permanganate can also be used as a short-term bath at concentrations of 10 mg/L for 30 minutes. At this concentration, careful observation of the fishes is mandatory to avoid mortality. This is a convenient treatment when fishes are being removed from ponds and brought out for sorting and shipping. It is particularly effective in minimizing columnaris infections after handling. Potassium permanganate can be used as a surface disinfectant at concentrations of 10 (30 - 60 minutes contact time) to 500 mg/L (30 seconds contact time) in a fish room or hatchery. Potassium permanganate will kill bacterial, fungal and many parasitic agents, but it is not viricidal.

Poor efficacy of potassium permanganate is usually caused by one of the three factors: (i) incorrect or incomplete diagnosis; (ii) incorrect calculation or measurement of amount of chemical needed; and (iii) excessive organic material in the system resulting in rapid degradation of the chemical. At any time when treatment failure occurs, sick fish should be brought to a diagnostic laboratory for accurate diagnosis. Measurement of the volume of water treated, accuracy of calculations to determine treatment rate and accurate measurement (by weight) of chemical used are essential for delivery of an appropriate chemical dose. As mentioned earlier, an excessive amount of organic matter in the system will result in rapid deactivation of potassium permanganate and, therefore, contact time with active chemical will be inadequate for effective treatment. This is often a problem in heavily stocked ponds.

***7. Metabolism of the drug in the animal body, retentivity and mode of excretion***

As mentioned above, potassium permanganate is an indiscriminate oxidizer, and as such can burn gill tissue and mucus of treated fish if too much chemical is applied. A rule of thumb to prevent excessive damage to fish is to avoid treating them with potassium permanganate more than once a week.

***8. Residue in the host species***

No residual effect has been reported.

***9. Host species safety***

Safe at the level stipulated.

***10. Environmental safety***

As with any other disinfectant it is harmful to many non-targeted plants and animals. However, the compound gets very easily deactivated in water rich with organic matter.

***11. Operator safety***

Potassium permanganate is fairly safe to handle, however, all chemicals should be treated with respect. Potassium permanganate will easily stain clothing and skin. Brown discoloration of skin is not painful, but it may be unsightly and takes several

days to disappear. Brown stains to clothings can be permanent. Protective gloves and clothings are recommended when handling potassium permanganate. Fish/shrimp farmers and aquarists do occasionally mix chemicals. It is important that formalin and potassium permanganate are NEVER mixed, as the combination can be explosive.

**6. Copper compounds**

Copper compounds are the only chemicals so far approved by USFDA for shrimp culture and are the oldest and most widely used chemicals in fish culture. They are used as a parasiticide against external protozoans in fishes. If copper compounds are used continuously in shrimp ponds, copper may accumulate at the bottom, attaining dangerous proportions.

A variety of copper compounds has been used as therapeutic and prophylactic agents for fishes in both fresh and salt water. Copper may account for 50 % of the disease treatment products available to saltwater hobbyists. Unfortunately, the efficacy of copper therapy is not well documented, even though treatment techniques have not changed appreciably in the last 20 years. There is, however, confidence in the efficacy of copper against protozoans, and even some metazoan parasites.

It is critically important to understand the interaction of copper with various ions in water to understand why a treatment that might be suitable for marine tropical fishes could be devastating in a freshwater system. Anything that affects the solubility or binding strength of copper complexes will affect bioavailability and bioactivity. The toxicity of copper depends on its speciation. Free cupric ion is most toxic, with complexed forms being less toxic. In natural seawater, copper is complexed with organic and inorganic ligands.

Dissolved copper can chelate with hydroxides, carbonates, amino acids, and polypeptides to form neutral, anionic, or cationic complexes in marine waters. Ammonia or amines generally form positive complexes with copper. A less stable neutral complex is formed between copper and acetate. In marine water, chloride ions bind strongly to copper ions and form stable divalent negatively charged soluble complexes that are less avaliable to biological systems than the hydrated complexes of copper ions. These chloride-copper complexes will break down at the alkaline pH of most seawater, causing copper to precipitate out in the form of insoluble salts and copper carbonate.

Copper depletion occurs owing to equilibrium with any calcium or magnesium carbonate present in a tank, such as coral, oyster shell, or dolomite. Changes in system dynamics, such as increased salinity or decreased pH, can redissolve precipitated or adsorbed copper suddenly, resulting in toxic levels.

**Toxicity**

Copper causes toxic changes in the nervous system, gills, liver, kidneys and the immune system of fishes. Fish exposed to acute overdoses of copper become dark and lethargic. At this stage of copper toxicity, gill lesions consist of blunting of the gill filaments with the presence of thick, dilated mucous cells and congested capillaries. With further

exposure, fish become indifferent to external stimuli and are easy to catch. If exposure continues, fish become uncoordinated losing normal posture. Finally, they become moribund and die.

As the toxicity progresses, serum potassium increases, probably due to cellular damage. In addition, exposure to even low levels of copper causes an increase in hematocrit, serum glucose, and serum levels of hepatic enzymes, as well as decreased immunoglobulin production. Hepatic lesions frequently consist of vacuolar degeneration. Direct effects of copper on lateral line mechano-receptors are documented, possibly explaining some of the postural changes seen.

Fish exposed to slowly rising copper levels can adapt themselves to the presence of the metal by inducing metallothionine production in the liver. This is the reason for the slow introduction of fish to therapeutic copper levels. Metallothionine binds copper and facilitates its internal storage without toxic effects.

**Antiviral compounds**

Viruses have two modes of transmission *viz.*, vertical and horizontal, either in latent or in virulent form. Therefore, antiviral preparations can be two types: those which do not permit a latent virus to express itself manifesting the disease, and those which either block viral binding sites to target cell receptors or as one step forward the ones which disrupt the synthesis of viral proteins or nucleic acid in host cells. There can be antiviral molecules, which prevent the cell burst and release of the virions. Several compounds under the broad category of **carbohydrates** (sulphated polysaccharides), such as Fucoidan, Carrageenan, Calcium spirulan, Dextran sulphate and Galactan sulphate, **lipids** such as sulpholipids, **proteins** such as cyanovirin-N are sourced from different families of algae, bacteria and sponges and reportedly possess antiviral activity. However, very little is known on the mode of action. Only a couple of compounds/drugs have been reported against aquaculture pathogens like Infectious Pancreatic Necrosis Virus (IPNV), Infectious Hematopoetic Necrosis Virus (IHNV), Yamane Tumor Virus (YTV) and White Spot Syndrome Virus (WSSV). Therefore, much work needs to be done to develop aquaculture grade antiviral drugs as therapeutic and prophylactic agents against viral infections.

**Natural antiviral molecules**

1. **Carbohydrates (Sulfated polysaccharides)**

   **A. Fucoidan:** Fucoidan is a mucilaginous intercellular polysaccharide with high viscosity, containing 30-70 % L-fucose and other sugars occurring together with alginic acid and laminarin in brown algae of the family, Fucaceae. Fucan extracts are structurally heterogeneous, with high molecular weight composed of 3-branched (1-2) or (1-3) linked alpha-L-fucose-4 sulphate unit. They exhibit numerous biological properties possessing anti-thrombic, anti-inflammatory and antiviral activities. The inhibitory effect of fucoidan on the replication of enveloped virus (herpes simplex virus), human cytomegalovirus and human immunodeficiency virus has been proved.

Extracted partially purified fucoidan (71.3 %) from the brown algae, *Cladosiphon okamuranus* was used to prepare shrimp diet. A high survival rate (78-82 %) was obtained among shrimps challenged with WSSV by the water-borne method following four days of feeding the diet. Reports of anti WSSV activity of fucoidan isolated from *Sargassum polystum* in *Penaeus monodon* has also appeared in literature.

**B. Carrageenan:** Carrageenan is a sulphated polysaccharide extracted from carragenophytes. Carragenates are defined salts of carrageenic acid. Carrageenan is a highly sulphated hydrocolloid and of different types. The important ones being κ-carrageenan, λ-carrageenan and ι-carrageenan. The sulphate content in κ-carrageenan is 24 - 28% and λ-carrageenan 24 - 33 %. The original source of the material was the Irish moss *Chondrus crispus* (red algae), but it is now obtained from a number of genera. Carrageenans were also isolated from the marine bacterium *Pseudomonas carrageenova***.** Carrageenan is co-internalized into cells infected with herpes simplex virus (HSV) inhibiting the virus and interfering with fusion (syncytium formation) between cells infected with the virus and inhibits the retroviral enzyme reverse transcriptase.

**C. Calcium spirulan:** A sulphated polysaccaride named calcium spirulan (Ca-Sp) with anti-viral activity has been isolated from the marine algae *Spirulina platensis* (Cyanobacteria). This polysaccharide is composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, gluconic acid, galacturonic acid, sulphate and calcium. It was found to inhibit the replication of several enveloped viruses, including herpes simplex virus type 1, human cytomegalovirus, measles virus, mumps virus, influenza A virus and HIV-1. Calcium spirulan seems to selectively inhibit penetration of virus into host cells. Retention of molecular conformation by chelation of calcium ions with sulphate groups was suggested to be indispensable to its antiviral effect.

**D. Dextran:** Dextran sulphate, obtained from red algae has been reported to inhibit syncytium formation acting external to the cell wall. There is evidence that dextran sulphate may be of some use clinically.

**E. Sulphated galactans:** A galactan sulphate (GS) isolated from aqueous extract of the red sea weed, *Aghardhiella tenera* inhibits the cytopathic effect of HIV-1 and HIV-2 in MT-4 cells at concentrations ten folds higher than those required for inhibition by dextran sulphate. The sulphated galactan isolated from the red sea weed *Pterocladia capillaceae* has proven to be active against HSV-1, HSV-2, human cytomegalovirus and pseudorabis virus. Sulphated xylogalactans have been isolated from red seaweed *Nothogenia fastigata,* which inhibits replication of HSV-1 and 2, establishing that the presence of sulphate group in the molecule is essential for its antiviral properties.

**2. Lipids (Sulpholipids)**

Sulpholipids (sulfonic acid containing glycolipids) extracted from cyanobacteria (blue-green algae) were found to be remarkably active against HIV. Sulphoglyco-

lipid in blue-green algae (*Spirulina sp.*) has been identified as a reverse transcriptase inhibitor of HIV.

The pure compound was active against HIV-1, cultured in human lymphoblastoid, CEM, MT-2, LDV-7 and C3-44 cell lines determined by the tetrazolium assay as well as gp24 viral protein and syncytium formation assay. Sulpholipids from blue green algae inhibit lipid-enveloped viruses including HHV-6A.

3. **Proteins**

**Cyanivirin-N:** Cyanovirin-N, is a potent protein extracted from certain strains of blue green algae (*e.g. Spirulina platensis, Nostoc ellipsosporum,* etc). It inhibits HIV, HHV-6, etc., by blocking the gp120 viral envelope protein and thereby binding to target T-cells. It also blocks the binding action of other lipid-enveloped viruses.

**Natural antiviral substances against fish viruses**

Many investigators have reported that survival of viruses in natural waters is influenced by extracellular compounds released by ambient microorganisms and several were reported to be responsible for viral inactivation in aquatic environments. Ammonia is a low molecular weight and heat-stable viricidal agent in activated sludge. A low molecular weight peptide (46 NW-O4 A) has been purified with antiviral activity composed of nine amino acids bound at its N-terminal with 3-hydroxy deconoic acid. However, most other investigators suggest that viral inactivation is caused by high molecular weight and heat labile substances produced by bacteria, such as proteolytic enzyme or ribonuclease.

A large number of bacterial strains from aquatic environment with antiviral properties have been screened and have demonstrated their inhibition of infectious hematopoietic necrosis virus (IHNV). The antiviral substance produced by one of the strains, *Pseudomonas flurescens* - 6 NW-04, was purified and identified as a peptide and demonstrated their direct inactivation or inhibition of viral invasion into CHSE-241 cells. Another compound (48 HS-21) isolated and purified from the culture supernatant of a marine *Alteromonas* sp. was active against many fish viruses like fish herpes virus (OMV), fish rhabdoviruses, IHNV, etc. The antiviral properties of crude extracts of *Rhodopseudomonas capsulata* on fish viruses such as IPNV, IHNV and YTV are well known. Antiviral activity of several Thai traditional herb extracts against fish pathogenic viruses such as IHNV, OMV and IPNV have also been reported.

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CHAPTER 2

# Anti-Parasitic Agents in Aquaculture*

Protozoan and metazoan parasitic diseases have considerable impact on fish production. Therefore, control and prevention of parasitic infection is of utmost importance in the success of aquaculture. Although chemotherapy is the last option in this direction, many a time, especially in the brood stock development, it is an inevitable ingredient. In this background, this chapter attempts to enlist the common anti-protozoal and anti-metazoan agents used in aquaculture, their specific use, mode of action and implication on the host and the environment. The anti-parasitic agents can be broadly classified as systemic anti-protozoal agents, externally applied anti-protozoal agents, metazoan ectoparasiticides and anthelmintics.

## A. Systemic anti-protozoal agents

### 1. Fumagillin

Fumagillin is produced by the parasitic fungus *Aspergillus fumigatus* and has low antibacterial and antifungal activities. However, the compound is active against protozoans.

#### *1. Structure and mode of action*

Fumagillin is acidic and is normally present as the dicyclohexylamine (DCH) $(C_6H_{11})_2$ NH salt. It acts by inhibiting RNA synthesis.

#### *2. Mode of application*

The Fumagillin DCH salt is sparingly soluble in water but moderately soluble in ethanol. It is heat-labile and cannot be incorporated in feed before pelleting. The drug may be surface coated by dissolving it in 95 % ethanol and spraying the solution onto the feed pellet.

#### *3. Anti-parasitic effect*

Fumagillin DCH has been found to be effective against microsporean and myxosporean parasites of fishes. Prevention of development of swim bladder inflammation of carp caused by *Sphaerospora renicola* has been reported with a diet containing 0.1 % Fumagillin DCH fed at 1 % for 4 months (10 mg/kg/day) to control whirling disease, an economically important disease caused by *Myxobolus cerebralis*. Fumagillin DCH at 10 mg/kg/day for 7 weeks was also found to be active against proliferative kidney disease. It has been reported that *Loma salmonae*, a microsporean parasite, which affects the gills of freshwater and marine fish, has been effectively controlled by feed medication (10 mg/kg/day) by Fumagillin DCH. Fumagillin DCH at 250 mg/kg/day for 30 days is reported to prevent *Pleistophora anguillarum* infection in Japanese eels.

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### *4. Target species safety*

Safety margin of Fumagillin DCH is reported to be narrow. Its main effects are on the erythropoietic system including reduction in the size of the spleen and kidney after prolonged treatment. Vacuolization of renal epithelial cells, anorexia and resultant retardation in growth have also been recorded in higher doses. In some species decrease in haemoglobin, haematocrit and erythrocyte count without affecting the haemopoetic tissue was reported. However, mortality was reported only at highest dosage and in the final stage of treatment, suggesting a cumulative toxic effect of fumagillin DCH.

### *5. Environmental safety*

There are no reports of residual effect of fumagillin DCH mixture in fish after treatment. However, if the drug is used in juvenile stage of the fish, concentration of residue would be little in 2-3 years old marketable size fish.

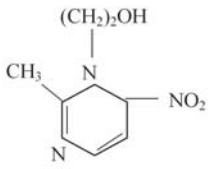
## 2. Nitroimidazole

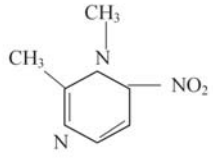
Nitroimidazoles are a group of synthetic antimicrobial agents active against protozoan parasites. Dimetridazole (DMZ) is one of the drugs used in the treatment of fish.

### *1. Structure and mode of action*

Information on the mode of action of the drug against protozoan parasites is not available. However, mechanism of action is thought to involve interference with DNA by the metabolites into which the nitro group of DMZ has been reduced.

*Dimetridazole*




*Metronidazole*

### *2. Anti-protozoal effect*

DMZ has been found to be effective against white spot /ich caused by *Icthyophtherius multifilis* in various food fishes. DMZ has been used as in-feed medication in combination with immersion treatment with $KMnO_4$. However, DMZ at a dose of 28 mg/kg/day for 10 days has been found to be equally effective without $KMnO_4$ immersion. DMZ has been found to be effective against hole-in-the-head disease caused by *Hexamita* and velvet disease caused by *Oodinium* by dip treatment at 5 ppm and 10 ppm, respectively.

### *3. Mode of application*

DMZ can be administered either through feed or through bath treatment.

### *4. Target species safety*

Information on the safety of DMZ on fish is not available.

***5. Environmental safety***

Elimination and half-life of nitroimidazoles is estimated to be eight hours in higher animals. It has also been recorded that in fish DMZ enters musculature and is eliminated quickly.

## B. Externally applied anti-protozoal agents

### 1. Formalin

Formalin is a versatile chemical with anti-parasitic, prophylactic and disinfecting activities. It is widely used against many protozoan parasites on skin and gills of fish.

***1. Structure and mode of action***

Formalin is a saturated solution (34-38 % W/W) of formaldehyde with methyl alcohol as stabilizing agent to delay polymerization. Formalin has a deoxygenating effect and the oxidation product of formaldehyde is formic acid. The polymerization product, paraldehyde is highly toxic to fish and any polymerized product, which appears as white precipitate, should be filtered out before application. Polymerization occurs faster in sunlight and at low temperature. Therefore, formalin should be stored in dark at a temperature above 10 $^{o}$C.

***2. Anti-parasitic effect***

Formalin is effective against skin or gill infecting protozoans such as *Chilodonella*, *Epistylis, Ichthyododo (Costia) necater*, *Icthyophthiirus multifiliis*, *Scyphidia* and *Trichodina*. It is also useful against the monogenetic trematode infecting gills, *Dactylogyrus* and skin infecting *Gyrodactylus* and also flukes such as *Cleidodiscus* and *Diplectanum*. It is also effective against peritrichous ciliate infection in cultured marine shrimp.

***3. Mode of application***

The normal treatment level of formalin is 1:6000 to 1:4000 (167-250 ppm) for up to 1 hour (17-25 ml/100 L for 30-60 minutes). Formalin is also used as dip (200 ppm) or as low dose exposure treatment in closed systems such as ponds and tanks at 2 ml/100 L for 12 h. Considering the deoxygenating effect of formalin, aeration should be provided during the entire period of treatment.

***4. Target species safety***

It has been found that different species of fish have different levels of tolerance. However, the adverse effect of formalin is found in the gill epithelium and the sign of toxicity is hypoxia.

***5. Environmental safety***

When formalin is used in culture system with re-circulating water supply, it would adversely affect the nitrifying bacteria in the biofilter. Further, formalin would also kill phytoplankton in the culture system and this would decay and increase the oxygen

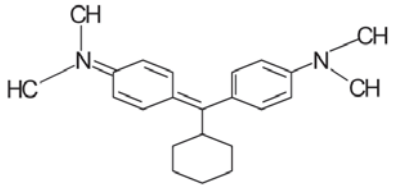
demand. Formalin is also harmful to the operators and, therefore, contact with the skin and inhalation of formaldehyde fumes should be avoided.

**2. Malachite green**

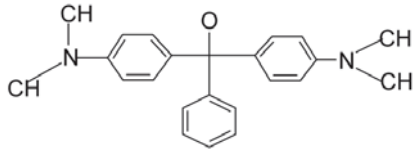
Malachite green is an effective therapeutant used for the control of various ectoparasites of freshwater and marine fishes. It is also used in the treatment of some endoparasite and as a prophylactic and disinfectant.

***1. Structure and mode of action***

*Malachite green*




*Carbinol*




Malachite green ($C_{23}H_{12}N_2$) is a synthetic organic dye having the colour of copper containing mineral, malachite, but contains no copper. Malachite green, in the presence of water, exist as an equilibrium between two forms: the dye which is soluble in water and water insoluble carbinol, a colourless non-ionic base. The carbinol, which is more liposoluble, can penetrate the biological membrane and enter the cells. This is believed to bind strongly with the internal cytoplasmic structures of parasite and interfere with the normal metabolism.

***2. Anti-parasitic effect***

Although malachite green is basically an antibacterial and antifungal agent, it has been found to be effective against proliferative kidney disease (PKD) in salmonids. It could also be used against similar disease caused by *Mitraspora cyprini* in gold fish.

***3. Mode of application***

It is found that both the efficacy and toxicity are dependent on concentration and time. In the case of PKD, a schedule of three treatments of 1 ppm at 4-7 days interval between the treatments has been reccommended. To control ectoparasites, it has been recommended to apply 1 ppm malachite green for up to 1 hour. Long-term (6 days) bath treatment (0. 2 ppm) has also been reported

***4. Target species safety***

Malachite green is reported to be toxic to fish and toxicity depends on water condition. Generally, it is more toxic and less effective in alkaline condition. Malachite green is reported to damage gill epithelium and thereby impair respiration. It is also reported to inhibit the iron containing enzymes and the cytochrome oxidase system in the mitochondria. There are also reports about the mutagenic potential of malachite green when used to treat trout eggs.

5. ***Environmental safety***

In the environment malachite green will be converted into microbiologically inactive carbinol and absorbed onto organic particles and subsequently be destroyed by oxidation.

**3. Leteux-Meyer mixture**

Considering the limited efficacy of either formalin or malachite green separately, at concentrations which are less toxic for fish, Leteux-Meyer (1972) found that a mixture of malachite green and formalin could be effective in the treatment of parasites with adequate target species safely.

1. ***Structure and function***

Leteux-Meyer mixture is formulated as a concentrate by dissolving 3.3 g malachite green in 1L formalin. It is recorded that the action of malachite green-formalin mixture is not simple additive, but is synergistic.

2. ***Anti-parasitic activity***

Leteux-Meyer mixture is found to be effective in treating ichthyophthriasis, *Costia* (*Ichthyobodo*), *Trichodina, Chilodonella, Scyphidia* and *Trichophyra*.

3. ***Mode of application***

Leteux-Meyer mixture is used as diluted solutions; 1:40000 (25 ppm) of formalin and 0.083 ppm of malachite green, for 1 h or 1:67000 (15 ppm of formalin and 0.05 ppm of malachite green) for prolonged treatment.

4. ***Target species safety***

Since the individual concentrations of formalin and malachite green separately in Leteux-Meyer mixture are less than the tolerance limit of many fishes, toxic effects have not been reported.

5. ***Environmental safety***

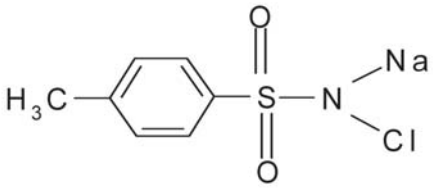
Information on this aspect is not available. However, it has been recorded that if formalin is used separately for treatment, the concentration which is safe for the target species needs to be used repeatedly. However, this could lead to environmental problems. A similar problem is anticipated with the malachite green also, if it is used separately at a higher concentration.

**4. Chloramine – T**

Chloramine – T is one of the most useful chemicals available for use in aquaculture. It is used as disinfectant, and also used as antimicrobial and anti-protozoan chemical. In comparison with formalin, Chloramine – T has greater efficacy against bacteria but much lower against protozoa. Chloramine – T is considered to be a safer disinfectant than chlorine because chlorine combines with organic matter to form carcinogenic trichloro-methane.

1. ***Structure and function***

Chloramine – T is a sodium salt whose anion slowly decomposes in water to the hypochlorite anion and hence to weak hypochlorous acid (HClO). This subsequently decomposes to chlorine and oxygen. Hypochlorous acid is a strong disinfectant, but hypochlorite ion has less disinfecting property. This is the reason why the activity of Chloramine – T is greater in acid conditions. Degradation product of Chloramine – T is p-Toluene-sulfonamide (pTsa).




2. ***Anti-parasitic activity***

Chloramine – T is effective against *Costia, Trichodina, Ichthiophthirius* and *Gyrodactylus* and other related parasites. It is also used against bacterial gill diseases caused by mixed infection of bacteria and protozoa.

3. ***Mode of application***

In general a low dosage bath treatment is recommended. Two ppm of Chloramine – T applied repeatedly for 3 times at 4 h intervals to achieve a 12 hour exposure has been found effective. For dip treatment, 8.5-10 ppm of Chloramine – T for 1 hour daily for 5 days is recommended. Alternatively, lower concentration of 5-6.5 ppm is used for 3 hours daily.

4. ***Target species safety***

Toxicity of Chloramine – T to the target species is reported to increase in soft water and low pH. It has also been reported that Chloramine – T is less toxic at low temperature and is not found to accelerate the onset of symptoms of toxicity. Application of Chloramine – T at high dose dip will cause serious gill damage. Chloramine – T primarily affects gill epithelia and causes symptoms of hypoxia. Hyperplastic thickening of the lamellae, intercellular oedema and epithelial erosion are reported. In all doses of treatment, a reduction in growth has been recorded when compared to untreated controls.

5. ***Environmental safety***

Chloramine – T is poisonous if contacted with human skin or eyes. Records of other environment and non-target species safety are not available.

**5. Copper sulphate**

Copper sulphate is used in the treatment of ectoparasitic infections, especially in marine aquaria. It is also used in the control of bacterial gill diseases. It is not recommended for treatment of freshwater fishes.

1. ***Structure and mode of action***

   Copper sulphate ($CuSO_4$. $5H_2O$) is shown to have a strong activity and is effective in the removal of mucous from gills by literally shrinking the mucous cells.

2. ***Anti-parasitic effect***

   $CuSO_4$ can be used to treat a range of parasites infecting marine aquarium fishes, protozoan parasites such as *Cryptocaryon* (marine ich), *Trichodina, Amyloodinium* (marine velvet disease) as well as monogenean flukes, *Dactylogyrus* (gill flukes), *Gyrodactylu*s (skin flukes). It is also used to treat chyophtheriasis infection in gold fish.

3. ***Mode of application***

   $CuSO_4$ is administered by immersion and prolonged exposure. For long-term exposure in marine fish, a copper concentration of 0.1 mg/L is recommended. The normal procedure to achieve this concentration is to prepare a stock solution of 400 mg/L ($CuSO_4$. $5H_2O$). The stock solution is used at 1ml/L. If the level of copper becomes very high, water exchange should be carried out. A level of 0.1-0.2 mg/L should be maintained for at least 10 days.

4. ***Target species safety***

   Copper sulphate is reported to have an adverse effect on some fish species. Sub-lethal concentration of $CuSO_4$ will render the fish more susceptible to infections. $CuSO_4$ is potentially very toxic in soft water having low pH. It has also been recorded that copper ions will be absorbed over the whole period of exposure and levels of copper will be very high in liver and kidney.

5. ***Environmental/species safety***

   Copper sulphate is found to be lethal to marine invertebrates and elasmobranchs.

**6. Potassium Permanganate**

Potassium permanganate is generally used in the treatment of protozoan and monogenean parasites. It is also used in treating bacterial gill disease.

1. ***Structure and mode of action***

   Although potassium permanganate ($KMnO_4$) is widely used in the treatment, information on its mode of action is not available.

2. ***Mode of application***

   $KMnO_4$ is always used either as bath treatment or dip treatment. It has been used at 2 ppm in ponds as a prolonged bath against ectoparasites. In other cases, a treatment of 500 mg/100 L for 1 hour, repeating if necessary for 2-3 days, has been recommended. Alternative method of administration is through dip treatment (1000 ppm) for a short duration (10-40 second). If the organic load is high, treatment should be repeated after 24 hours.

3. ***Anti-parasitic effect***

   $KMnO_4$ is active against protozoan ectoparasitic diseases such as icthyotodosis (costiasis), ichthyophtheriasis and also against monogenean parasites.

4. ***Target species safety***

   $KMnO_4$, if applied in muddy water, will be neutralized completely and become ineffective. The compound may lead to toxic effects due to the deposition of manganese dioxide on the gills. Sufficient care should be taken in giving treatment to scale-less fishes and warm water fishes.

**7. Sodium chloride**

Sodium chloride is a versatile compound, which has been used for a variety of uses by fish farmers. Apart from its antifungal effect, it is being used in the treatment of bacterial gill disease and treating hatchlings with yolk. It is used for cleaning the muddy flavour from some fishes and also to release osmotic stress.

1. ***Structure and mode of action***

   Sodium chloride (NaCl) has a mild and a stringent effect and because of this property it helps in lifting off the mucus from the gills.

2. ***Mode of application***

   Sodium chloride is applied as a bath/dip treatment. In small fry, a treatment dose of 0.5 % (5000 ppm) for 30 min or 1 % (10000 ppm) for 6-10 minutes is recommended. Another method of treatment is to expose the fish in a gradient of salt concentration. This can be carried out by placing a pile or bag of salt at the bottom of the trough or pond. In this situation fish may follow the salt concentration and swim closer to the bag, where it will be exposed to an adequate dose of salt.

3. ***Anti-parasitic effect***

   Sodium chloride is generally used in the treatment of ectoparasite such as *Costia, Chilodonella, Ichthyopthirius,* particularly in fry stage.

**C. Metazoan ectoparasiticides**

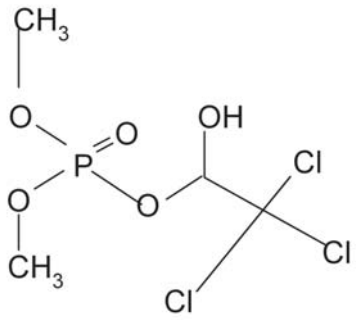
**1. Organophosphorous compounds**

Organophosphorous compounds are widely used in aquaculture for the control of sea lice, a crustacean parasite. They have been used to treat other metazoan parasites as well. There are three organophosphorous compounds which are commercially available and widely used in aquaculture - Trichlorfon, Dichlorvos and Azamethiphos.

**2. Trichlorfon**

Trichlorfon was the first organophosphorous compound to be developed for the control of sea lice.

1. ***Structure and mode of action***




As an organophosphorous compound, trichlorfon acts as an inhibitor of neuromuscular transmitter. It inhibits the enzyme, acetyl-cholinesterase (AchE), which is responsible for the hydrolysis of acetylcholine, the neuromuscular transmitter in both vertebrates and arthropods. Inhibition occurs in fish when the compound is ingested, whereas in arthropods it acts as a contact poison because of the ready penetration of this compound through cuticle. In vertebrates, organophosphorous compounds are hydrolysed rapidly so that the AchE regenerates and hydrolyses the accumulated acetylcholine. However, since arthropods do not have the enzyme to hydrolyse this compound quickly, inhibition of neuromuscular transmission and the resultant muscular contraction will be prolonged.

2. ***Mode of application***

The brand of insecticide 'Neguvon', which consists of 97 % trichlorphon and 3 % stabilizers is available and is being used in aquaculture. Initially, it was administered orally. However, due to low target species safety, it has been suggested to apply it as bath. The dose of trichlorfon in fish against monogeneans is 0.25mg/L. In certain cases, a combined treatment of trichlorfon with mebandazole is used. A dose at the rate of 0.4 mg/L mebandazole and 1.8 mg/L trichlorfon has been suggested in freshwater. The normal level of trichlorfon is 0.2 ppm active ingredient. In the treatment of *Argulus*, following dosages are recommended: 6.25 ppm for 60 min; 12.5-50 ppm for 30 min; 100 ppm for 10 min.

3. ***Anti-parasitic effect***

Trichlorfon is effective against sea lice, *Lepeophtheirus salmonis,* and also against gill and skin infecting monogeneans, *Dactylogyru*s and *Gyrodactylu*s. It is also reported to be effective against *Argulus* and *Lernaea*. In the treatment of sea lice, it has been found that the compound is effective only against the last three or four life stages known as pre-adults and adults.

4. ***Target species safety***

In water, trichlorfon will be gradually converted into dichlorvos. Dichlorvos is about 100 times more potent AchE inhibitor than trichlorfon. Further, it is more lipid-soluble and hence more readily absorbed. Therefore, the toxicity and efficacy of trichlorfon depends on the rate at which it is converted to dichlorvos. This in turn significantly depends on the temperature, pH, sunlight and aeration in the water.

5. ***Environmental safety***

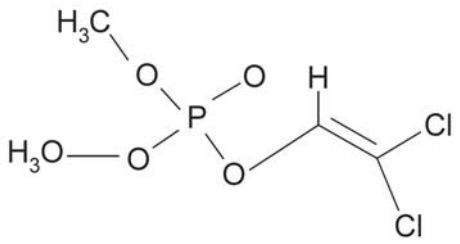
Reports indicate that residue of trichlorfon is not persistent. A withdrawal period of 21 to 30 days has been approved for this compound.

### 3. Dichlorvos

Dichlorvos has almost become a standard material for the treatment of sea lice, *Lepeophtheirus salmonis* and *Caligus*. 'Nuvan', the insecticide formulation of dichlorvos, was the first to be tried in aquaculture and a specific formulation, 'Aquaguard SLT' (sea lice treatment) was developed. This is an emulsifiable liquid containing 50 % w/v dichlorvos, 8 % emulsifiers and 42 % di-n-butyl phthalate (DBP).

#### *1. Structure and mode of application*

The generally recommended dose is 1 ppm dichlorvos (2 ppm Aquaguard SLT) treatement for 30-60 minutes. However, there are many limiting factors which determine the actual efficacy, such as mixing of the compound in a uniform manner, temperature and aeration.




#### *2. Anti-parasitic affect*

Dichlorvos is more potent than trichlorfon and due to its lipid– soluble nature it is more rapidly absorbed. It is effective against *Lepeophtheirus, Caligus, Lernaea, and Argulus,* and also against monogenean parasites.

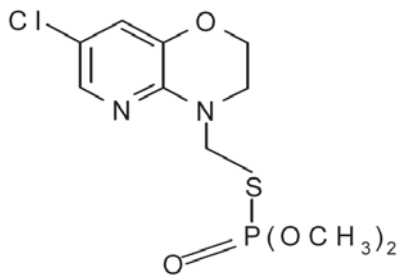
#### *3. Target species safety*

There are reported toxic effects of dichlorvos on target species. These include muscular rigidity and congregation of fish at the bottom of the tank/netpen and falling on the sides. The smaller fishes are found to be more susceptible. Since the active inhibition prolongs for many days, repeated treatment is risky. It is also found that oxygen deficiency enhances the anti-AchE activity of dichlorvos.

#### *4. Environmental safety*

Studies on persistence of residues in treated fish are limited. Since the residue of dichlorvos persists for one or two weeks, the treatment cannot be recommended for fishes which are about to be harvested. Dichlorvos involves operational hazard to users as the compound is neurotoxic and can be absorbed by inhalation, ingestion and through the skin. Dichlorvos also involves non-target species safety hazard. As it is toxic to arthropods, and since it is released into the environment after use, it is a potential hazard to invertebrate wild life and farmed aquatic invertebrates. Therapeutic dose of dichlorvos is found to be toxic to lobster larvae and adult, zooplankton and phytoplankton. Crustaceans appear to be the most susceptible groups. However, molluscs such as mussels, clams, oysters were found to survive the therapeutic concentration.

## 4. Azamethiphos




Azamethiphos is the latest organophosphorous compound developed for the treatment of sea lice. It has many advantages over dichlorvos such as enhanced activity, more safety to the operator, as it is commercially available in wettable powder in water-soluble sachets and less toxic to mammals.

### *1. Structure and mode of action*

Similar to other organophosphorous compounds, azamethiphos also acts as an anti-cholinesterase agent. It has got the same spectrum of activity to different life stages of sea lice as that of dichlorvos with greater activity. This is commercially available as 'Salmosan'.

### *2. Mode of application*

Recommended concentration is 0.1 ppm and the salmosan is available as 50 % wettable powder in 20 g and 100 g water-soluble sachets. Initially, concentrated solution should be made by dissolving the powder in watertight container using distilled water. Before use, concentrated solution should be diluted in seawater. Exposure time is 60 minutes at temperature below $10^{o}$C and not more than 30 minutes above $10^{o}$ C.

### *3. Anti-parasitic effect*

Substantial cumulative mortality of sea lice has been observed in *in vitro* treatment. However, *in vivo* studies showed varying sensitivities.

### *4. Target species safety*

At 0.1 ppm, fish may show hyperactive response. A 0.3 ppm, repeated treatment experiment showed a significant AchE depression in brain. Sensitivity also varies with species of fish.

### *5. Environmental safety*

Similar to dichlorvos, azamethiphos at normal dosage is significantly toxic to crustaceans. Sensitivity of crustaceans also varies from species to species. It is found to be less toxic to crabs than shrimps and lobsters are found to be extremely sensitive. Owing to the low concentration of the compound used in the treatment and its rapid metabolism in fish, the withdrawal period approved for salmosan is 24 hours. Although it comes as wettable powder, azamethiphos still presents a significant hazard to the operator.

## 5. Hydrogen Peroxide

Hydrogen peroxide is proved to be an efficient therapeutant against sea lice, protozoans and flukes. However, the problem of recovery of sea lice and re-infestation in fish have been reported.

1. ***Structure and mode of action***

   Hydrogen peroxide ($H_2O_2$) is normally supplied as 35 % or 50 % aqueous solutions. Its effect on bacteria is believed to be due to its decomposition to hydroxyl radicals, and the effect on protozoans and flukes might be due to intracellular oxidation. Although the mode of action of $H_2O_2$ on sea lice is not entirely known, it is presumed that it causes gas/oxygen embolism in sea lice, which results in floating up of the lice.

2. ***Anti-parasitic effect***

   It is generally used in the treatment of sea lice. However, it is not found to be very effective against protozoans and flukes.

3. ***Mode of application***

   $H_2O_2$ is generally administered as bath. Since commercial concentration of $H_2O_2$ is extremely corrosive to fish tissues, it must be mixed into water safely. A recommended dose of 1.0 or 1.5 g/L of $H_2O_2$ bath for 20 minutes gives maximum removal of sea lice. A longer exposure of 30 minutes will also give effective results. Efficacy of $H_2O_2$ is related to concentration, but only over a narrow range. Although efficacy rises with temperature, the toxicity to the target species also rises steadily. Therefore, $H_2O_2$ is not recommended to be used in water above $14^oC$.

4. ***Target species safety***

   Toxicity of $H_2O_2$ to target species depends on many factors such as higher temperature, lower exposure higher concentration. The smaller fishes are more sensitive to $H_2O_2$ toxicity. Studies on the symptoms of toxicity showed that it ranges from initial escape response to fatality. Symptoms also include increased ventilation, congregating at the surface of water, settling at the bottom of the cage and loss of balance. Histologically, lesions are reported in the gills, epithelial hyperplasia and hypertrophy, resulting in lamellar fusion. In some cases, bleeding and necrosis of the epithelia takes place resulting in lifting of the gill epithelium.

5. ***Environmental safety***

   Since the aqueous solution (35-50 %) is highly corrosive to all tissues, any direct contact by the operator is hazardous. However, since $H_2O_2$ degrades to $O_2$ and water, it is considered safe both to the consumer (operator) and to the environment.

**6. Ivermectin**

Ivermectin is an orally administered ectoparasiticide, which has got many advantages of organophosphorous compounds and $H_2O_2$. Its merits include the fact that it can be administered orally without stressing the fish and unlike dichlorvos it is not toxic to vertebrates. Further, it has got increased safety margin and is less expensive when compared to $H_2O_2$.

1. ***Structure and mode of action***

   Ivermectin increases the production of the inhibiting neuro-transmitter, gama amino butyric acid (GABA) at nerve endings and enhances the binding of GABA to receptors. In invertebrates since this occurs in muscles as well as in synapses, ivermectin causes paralysis. However, in vertebrates GABA is confined to the central nervous system, and ivermectin does not easily cross the blood-brain barrier.

2. ***Mode of action***

   Complete dose titration on ivermectin has not been reported so far. However, as a standard treatment, 0.2 mg ivermectin per kg feed (mammalian dosage) is recommended. A treatment schedule of 0.005 mg ivermectin per kg feed given every third day for 3 to 6 times has significantly reduced the sea lice infection. The studies reported the use of commercially available 1 % oral drench diluted in deionized water and sprayed onto feed pellets.

3. ***Target species safety***

   Prolonged feeding of 0.2 mg/kg dosage of ivermectin is reported to cause mortality in fish. However, lower dosage does not lead to mortality. It has been reported that after 6 doses, feed intake dropped significantly. Toxicity symptoms include darkening of the skin and loss of equilibrium. These symptoms were recorded after the first dose at highest rates and after six doses at the lowest.

4. ***Environmental safety***

   Pharmokinetic study of ivermectin showed that it is not suitable for use in target species, mainly because of consumer safety. One of the demerits of ivermectin is its questionable environmental safety. It is toxic to a wide range of invertebrates.

**7. Pyrethroids (Cypermethrin)**

Pyrethrins are extremely photo-labile natural compounds extracted from plants of the *Pyrethrum* (Compositae) genus. However, pyrethroids are synthetic chemical analogues of pyrethrin possessing insecticidal properties.

1. ***Mode of application***

   Cypermethrin is a pyrethroid, which is being used against sea lice. Although in-feed administration was tested initially, it was found that bath administration would be the effective way, and subsequently a bath formulation, 'Excis' has been developed. It is a formulation of 1 % alcohol solution of cypermethrin with a biodegradable surfactant. It has been demonstrated that 5 µg /L (0.005 ppm) of Excis in seawater at $10^{o}$ C for 1 hr is effective in reducing the chalimus stage of sea lice significantly.

2. ***Environmental safety***

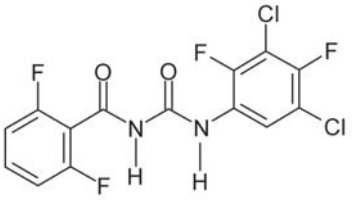
   Cypermethrin, after treatment, becomes adsorbed on to particulate and a lesser extent to dissolved organic matter. This reduces the preparation of cypermethrin in the water phase and thus reduces the uptake by fish. It has also been found that half-life

of cypermethrin is 78 hours and it has got a very short withdrawal period. It has also been reported that since the surfactant in 'Excis' is biodegradable, it enhances the diffusion and distribution of cypermethrin in water. At the end of treatment, due to dilution, the surfactant cannot hold the hydrophobic cypermethrin in suspension.

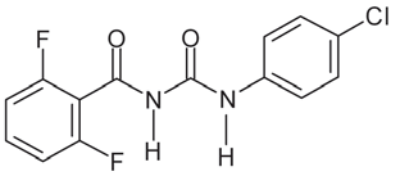
**8. Benzyl-ureas**

This is one of the latest groups of chemicals used in the control of sea lice. There are two commercial products available in this category: Diflubenzuron and Teflubenzuron.

***1. Structure and mode of action***




*Teflubenzuron*




*Diflubenzuron*

These chemicals normally interfere with the synthesis of chitin, the product from which the exoskeleton of arthropods is formed. Being an arthropod, in sea lice, ecdysis/molting marks the division between two developmental stages. Benzyl-ureas, at ecdysis, render the sea lice incapable of shedding old exoskeleton. However, benzyl-urea has no effect on arthropods at inter-ecdysis stage. Therefore, the chemical should be applied for a prolonged period of time to ensure the inclusion of all ecdysis stages.

***2. Mode of application***

The chemical is administered as medicated feed pellets. Teflubenzuron is commercially available as 100 % powder. This has to be mixed with feed at 2 kg per tonne. These pellets are fed at 0.5 % for 7 days (10 mg/kg/day).

***3. Target species safety and environmental safety***

No reports are available on these criteria. However, it has been reported that absorption of diflubenzuron is slow and incomplete.

**D. Anthelmintics**

Despite the fact that the range and severity of helminth infection is a serious problem in fish, none of the anthelmintic possess market authorization in either the European Union or the United States.

**1. Benzimidazoles**

There are a variety of benzimidazoles available for aquaculture use. However, studies showed that out of the nine commercially available compounds tested against skin fluke, *Gyrodactylus* sp., majority had no activity at concentrations below the 'no observable effect concentration (NOEC)'.

1. ***Mode of application***

   Mode of administration is through water or through feed. It has been reported that mebendazole and parabendazole at 25 mg/L for 12 hrs resulted in 90 % elimination of flukes' whereas fenbendazole showed 100 % elimination at the same dosage and high degree of activity seen even at 1.5 mg/L dose. Studies also indicate that although some gill and skin flukes are resistant to trichlorfon, they are susceptible to mebendazole and *vice versa*; a combination of 0.4 ppm mebendazole and trichlorfon for 24 hours has been found to be effective against both types of parasites. It has also been reported that feeding of fenbendazole at a rate of 40 mg/kg, twice at an interval of four days is effective against *Bothriocephalus acheilognathi*.

2. ***Target species safety***

   It has been reported that 100 ppm mebendazole can be tolerated by eels for 72 hours but 500 ppm can be lethal by 52 hours.

**2. Praziquantel**

Praziquantel is considered to be a standard drug for treatment of cestodes (tape worms) in fish and has been proved useful against trematodes as well.

1. ***Mode of action***

   Praziquantel is believed to impair neuromuscular system of the parasite and is found to inhibit the functioning of hooks and suckers on the scolex. It is also presumed to affect the permeability of the integument of the parasite leading to osmotic and nutritional imbalance.

2. ***Mode of application***

   Praziquantel is administered both as immersion treatment or as in-feed administration or intramuscular injection. In the immersion application it is found to be more effective when dissolved in dimethyl sulphoxide (DMSO) than in ethanol. In immersion treatment, it has been recorded that a concentration of 2 ppm for 2-4 h was effective in killing significant number of eye flukes. It is also found to be 100 % effective against monogenean flukes at a concentration of 600 ppm for short duration and at 120 ppm after 8 h exposure. It has been recorded that in-feed administration of Praziquantel at 330 mg/kg/day for 7 days appear to be the optimum therapeutic dose to treat eye fluke infection. *Ad libitum* feeding of medicated pellet at the same dosage for 15 days was found to give complete protection against artificial infection. Praziquantel was found to be effective against yellow grub disease at a dose of 2 ppm immersion. Another compound, nicolsamide is found to be active against *Gyrodactylus* at 0.1 ppm and appeared to have greater effect than praziquantel on immature parasites.

3. ***Target species safety***

   High feeding rate of the drug did not show any adverse effect on feeding. Other information on the toxic effect of the drug is not available.

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**Trade names of anti-parasitic drugs specifically formulated for fish (Treves-Brown, 2000)**

| Trade Name | Manufacturer *(Country)* | Active Ingredients |
|---|---|---|
| Aquagard | Novartis (UK) | Dichlorvos |
| Excis | Grampian (UK) | Cypermethrin |
| Fumaqua | Sanofi (France) | Fumagillin |
| Paramove | Solvay-Interox (UK) | Hydrogen peroxide |
| Paracide-F | Argent (USA) | Formailn |
| Parasite-S | Syndel (Canada) | Formalin |
| Protoban | Vetark (UK) | Formalin and Malachite green |
| Salartect | Brenntag (UK) | Hydrogen peroxide |
| Salmosan | Novartis (UK) | Azamethiphos |

CHAPTER 3

# Weedicides in Aquaculture*

In scientific shrimp/fish culture, eradication of unwanted animals and plants (weed) becomes an essential component as the cultured animals should not compete with them for space and food and most important is the possibility of eliminating potential carriers and reservoirs of infection from a system. This chapter deals with important weedicides, which can be applied to the ponds with least environmental damage.

## 1. Rotenone (Derris root)

1. ***Chemical name:*** Rotenone

   Rotenone is a white odourless substance in derris root, which inhibits respiration in fish.

2. ***Target organism***

   Against fish eggs and larvae for eradication.

3. ***Mode of action***

   Rotenone kills fish by blocking electron transport by the cytochrome system in gill tissue.

4. ***Mode of application (including recommended preparations required for applying in aquatic system)***

   Plant poisons are usually processed to increase their potency. The plant material is dried, then soaked in water overnight and pounded flat. The flattened fibers are dipped and squeezed in a pail of water until the water becomes milky. The milky liquid is broadcast over the pond surface.

5. ***Shelf life and storage***

   The derris root can be dried and maintained in a cool dry place for a period not more than one year. Such material has to be soaked overnight for use.

6. ***Recommended dosage***

   Most effective for eliminating fish eggs and larvae when applied at a concentration ranging from 5-10 ppm with an exposure period 6-24 hours. An aliquot of 2 ppm is toxic to some fish species, but for fish eradication, 0.05 to 0.5 ppm of active ingredient is normally used, depending on target species.

   When the commercial source material is used, a quantity of 20 kg of commercial powder containing 5-8 % rotenone is applied per acre of water with an average depth of 10 cm.

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7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

   No information is available on the metabolism of rotenone in animal body, its retentivity and mode of excretion.

8. ***Residues in host species***

   No information on its residue in the host body is avalaible.

9. ***Host species safety***

   Rotenone is used for eradicating weed fishes and the poisonous effect may last from 4 to 12 days depending on the dosage used. Therefore, time of stocking has to be regulated depending on the residual effect in water and sediment.

10. ***Environmental safety***

    Rotenone is much less toxic to bottom living invertebrates than fishes but zooplankton populations may take several months to recover from treatment. When used as a piscicide, rotenone breaks down in water within a period of two weeks or more depending on temperature. Fishes may be introduced only after this period. High temperature, high alkalinity and light exposure increases rotenone degradation.

11. ***Operator safety***

    Hazardous to operators as inhalation may result in respiratory paralysis. Prevent any liquid from getting into eyes.

## 2. Saponin

1. ***Source***

   The main source of saponin is tea seed (the residue from oil processing of the seeds of *Camellia* that contains 10-15 % saponin) and mahua oilcake.

2. ***Target organism***

   Saponin is particularly favored by shrimp farmers because it is toxic to fish but not to crustaceans. It also facilitates moulting in shrimp in small quantity. Used for the treatment of Black Spot disease in shrimp, for controlling algal fouling and the treatment of tail rot and protozoan infestations

3. ***Mode of action***

   Saponin destroys red blood cells of fishes.

4. ***Mode of application (including recommended preparations required for applying in aquatic system)***

   The required amount of tea seed cake is first crushed into small pieces and soaked in a tub or vat of water for about 24 hours. This mixture is then broadcast evenly over the pond surface having 5 to 10 cm water. In large ponds, the poison may be soaked overnight in a boat and spread over the pond the next morning.

In the case of mahua oil-cake, the residue after oil extraction is ground and the powder can be thrown into puddles or broadcast over the water surface.

***5. Shelf life and storage***

If properly maintained in air tight chambers in a cool dry place it can remain stable for atleast three months.

***6. Recommended dosage***

Saponin (20-30 ppm) has been recommended for the treatment of black spot disease in shrimp. Saponin is also effective in controlling algal fouling. Tea seed cake at 3.5-5 kg/1000 $m^2$ is promoted as treatment for tail rot and is used at 5-25 ppm for treatment of protozoan infections. Locally, it is applied at 2-3 ppm for 24 hours to stimulate moulting in shrimp. Tea seed cake at 5 ppm is used to remove unwanted fishes in shrimp culture ponds. It is used @ of 40 kg of commercial powder containing 6 % saponin per acre of water with an average depth of 10 cm.

***7. Metabolism of the drug in animal body, retentivity and mode of excretion***

No information is available on the metabolism of saponin in animal body.

***8. Residues in host species***

No information is available on the residual effect of saponin in host species.

***9. Host species safety***

During normal application no deleterious effects are noticed. Poisonous effects may last from 2 to 8 days depending on dosage used.

***10. Environmental safety***

The compound degrades rapidly, with reported half life time of a few days. However, subsequent to the application of saponin, the pond water should not be drained and instead it should be allowed to get degraded in the pond itself. Release of water from ponds freshly supplied with saponin may result in fish kill in the receiving waters.

***11. Operator safety***

Not hazardous to human beings.

**3. Ammonia**

***1. Chemical name and structure***

Ammonium sulphate ($NH_4$) 2SO4

Ammonium Chloride ($NH_4$ Cl)

Unionized ammonia is the specific component which kills weed fish and prawn.

***2. Target organism***

Aimed at eliminating weed fish and prawn.

### 3. *Mode of action*

Unionized ammonia passes across gill membrane of fishes and prawns and alters the pH of body fluid and cytoplasm leading to death of the animal. Increasing water pH by adding 50 to 100 kg quicklime per $m^2$ with approximately 10 cms water coloumn just before adding ammonium sulfate increases its efficacy by converting $NH_4^+$ into $NH_3$.

### 4. *Mode of application*

The required quantity of quick lime as per the ratio is first applied in water/sediment and subsequently ammonium sulphate/ammonium chloride is applied. In case ammonia gas is applied, it is to be bubbled through water from a cylinder.

### 5. *Shelf life and storage*

Ammonium sulphate/ammonium chloride being chemicals remain stable indefinitely provided they are stored in cool dry places.

### 6. *Recommended dosage*

For best results, ammonium sulphate/ammonium chloride must be applied along with lime in the ratio of 1:5 and the ammonia output ($NH_3$) should not be less than 10 ppm at any point of time. Therefore, the quantity of salt required has to be calculated based on the total quantity of water in the pond.

### 7. *Metabolism of the drug in animal body, retentivity and mode of excretion*

The chemicals are not known to be consumed. However, the unionized ammonia which penetrates the cell membrane may be excreted through the normal excretory pathway.

### 8. *Residues in host species*

No known residual effect has been reported.

### 9. *Host species safety*

Since ammonia is used as an eradicant, care has to be exercised in ensuring that not more than 0.01 ppm ammonia is used in grow out systems while stocking.

### 10. *Environmental safety*

No environmental hazard is expected as ammonia is easily nitrified to nitrite and then to nitrate and consumed by microorganisms and macro algae as nitrogen source.

### 11. *Operator safety*

Ammonia as gas is hazardous to human beings and, therefore, mixing of ammonium salt with lime must not be done before application. Those who operate ammonia cylinders must wear an oxygen mask.

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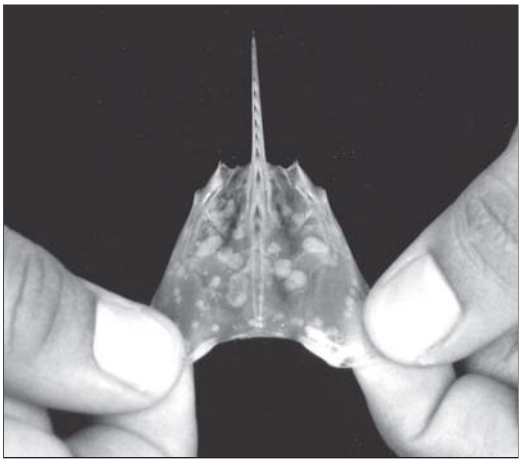

CHAPTER 4

# General Management Chemicals in Aquaculture*

Apart from several anti-microbials and anti-mentazoal compounds and weedicides for successful aquaculture, several management chemicals are also used worldwide. In most situations, on careful application, they are found to be useful in managing the system and several vital parameters which favour sustainability are maintained at optimum levels. This chapter deals with some of the important general management chemicals.

## 1. Alum

### *1. Chemical name*

Aluminum potassium sulphate ($AlK(SO_4)_2.12H_2O$)

### *2. Target organism*

As such not specifically targeted at any organism.

### *3. Mode of action*

Alum reacts with negatively charged clay particles causing them to coagulate into larger particles and settle them. Alum reduces turbidity by 89-97 % within 48 hours at doses of 10-30 mg/litre. Alum is acid forming and can substantially reduce total alkalinity and pH as per the following reaction:

$$Al_2(SO_4)_3 + 6H_20 = 2Al(OH)_3 + 6H^+ + 3SO_4^{2-}$$

This effect makes alum unsuitable for use in ponds with low total alkalinity as it may lower pH to the point where it is deleterious to fish.

### *4. Mode of application*

Added directly to water.

### *5. Shelf life and storage*

Stays indefinitely on storing in cool dry place.

### *6. Recommended dosage*

Used at concentrations of 10-20 mg/L as a flocculent.

### *7. Metabolism of the drugs in the animal body, retentivity and mode of excretion*

There is no known consumption of alum into the animal body.

### *8. Residues in host species*

No information is available on its residual effect.

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9. ***Host species safety***

   At the recommended concentration no toxicity is recorded.

10. ***Environmental safety***

    Alum reduces dissolved inorganic phosphate levels through the precipitation of insoluble aluminium phosphate, reducing phytoplankton growth. The use of alum therefore necessitates the addition of lime to correct total alkalinity and pH if these parameters fall below acceptable levels. Fertilization to increase dissolved phosphorus concentrations may also be necessary to maintain productivity.

11. ***Operator safety***

    Not hazardous to human beings.

## 2. EDTA

1. ***Chemical name and structure***

   Di-Sodium ethylene diamine tetraacetic acid ($C_{10}H_{14}N_2O_8Na_2.2H_2O$).

2. ***Target organism***

   EDTA is used as a treatment for ecto-commensal fouling by stimulating juvenile moulting.

3. ***Mode of action***

   Added to larval rearing water in shrimp hatcheries. It chelates divalent and trivalent metal cations. This will reduce the bioavailability of heavy metals by complexation. It is a chemical used to improve water quality by reducing heavy metal concentrations.

4. ***Mode of application***

   Added directly to water.

5. ***Shelf life and storage***

   Stays indefinitely on storing in cool dry place.

6. ***Recommended Dosage***

   In shrimp larval rearing, it is applied at 10 ppm prior to stocking of nauplii. It is also applied at 1-5 ppm to remove organic substances in the water column.

7. ***Metabolism of the drug in the animal body, retentivity and mode of excretion***

   No information is available on its absorption by animal body.

8. ***Residues in host species***

   No information is available on its residual effect.

9. ***Host species safety***

   At the recommended concentration no toxicity is recorded.

***10. Environmental safety***

As it is a chelating agent the only effect will be complexation with heavy metals that improve water quality. Therefore, it is an environmentally safe chemical.

***11. Operator safety***

Not hazardous to human beings.

## 3. Gypsum

***1. Chemical name***

Calcium sulphate ($CaSO_4$).

***2. Target organism***

As such not specifically targeted at any organism.

***3. Mode of Action***

Used as a flocculent in shrimp/fish ponds to reduce turbidity. Gypsum (calcium sulphate) is more soluble than liming materials and can be used to increase the total hardness of water beyond what is possible with lime, although it does not neutralise acidity. The low cost and high solubility of gypsum makes it ideal for use in the maintenance of calcium levels in hatchery situations. Adequate levels of calcium are critical for bone formation in fish and exoskeleton formation in crustaceans. The increased calcium concentrations associated with the addition of gypsum to ponds may cause a gradual, but substantial decline in total alkalinity, pH and phytoplankton abundance. The increased concentration of calcium associated with the addition of gypsum has been shown to reduce total alkalinity through the precipitation of calcium carbonate. High calcium concentrations have also been shown to limit the concentration of dissolved phosphorus through precipitation of insoluble tri calcium phosphate. The reduction in phosphorus in this manner is not normally enough to make ponds unproductive. However, as phosphorus concentrations are normally low in acidic waters due to ion exchange of sediments, the use of gypsum may therefore reduce the productivity of acidic ponds. It may be necessary to correct total alkalinity with lime and increase phosphorus levels with fertilization if treatment with gypsum causes these parameters to fall below acceptable levels. The calcium ions supplied by gypsum act as electrolytes in the flocculation of colloidal particles. For example, gypsum applied at the rate of 100-500 mg/L substantially has reduced turbidity in a study. However, similar reductions in turbidity were achieved using 20-25 mg/L of alum, which is a more cost-effective treatment.

***4. Mode of application***

It is added to the pond bottom before stocking if the lime requirement is established. Application in water during culture period is recommended in small doses as per pH and alkalinity.

5. ***Shelf life and storage***

   Stays stable indefinitly provided it is stored in a cool dry place.

6. ***Recommended dosage***

   In normal situation a concentration ranging from 20-25 mg/L will be sufficient to get the desired result. The additions can go up to 500 mg/L during times of high turbidity.

7. ***Metabolism of the drug in the animal body, retentivity and mode of excretion***

   The chemicals are not known to be consumed.

8. ***Residues in host species***

   No known residual effect.

9. ***Host species safety***

   During normal application no deleterious effects are noticed.

10. ***Environmental safety***

    No environmental hazards are expected.

11. ***Operator safety***

    Not hazardous to human beings.

## 4. Liming material

1. ***Chemical name and structure***

   Agriculture lime/limestone or crushed shell ($CaCo_3$)
   Hydrated lime or slaked lime ($Ca(OH)_2$)
   Quick lime/burnt lime or burnt shell lime (CaO)
   Dolomite or dolomite lime ($CaMg(CO_3)_2$)

2. ***Target organism***

   As such not specifically targeted at any organism.

3. ***Mode of action***

   Neutralizes acidity, increases total alkalinity and total hardness**.**

   $CaMg(CO_3)_2 + 4H^+ = Ca^{2+} + Mg^{2+} + 2H_2O + 2CO_2$

   Rapid pH changes, even within the range normally tolerated by a species, may also cause the death of fishes. Some liming materials such as calcium oxide and calcium hydroxide can result in a pH increase above 11, which is considered to be the alkaline death point for pond fishes. However, these materials will react with atmospheric carbon dioxide ($CO_2$) to form less hazardous carbonates if applied to empty ponds several weeks before refilling. This will prevent such excessive increases in pH occurring from the use of these materials. Ponds with substantial population of

phytoplankton or aquatic plants may also experience wide diurnal fluctuations in pH. This is caused by fluctuations in $CO_2$ concentration due to respiration and photosynthetic activity. The addition of liming material to increase the total alkalinity of ponds has the desirable effect of increasing buffering capacity and pH stability. Liming material are useful in precipitating suspended and soluble organic material, decreasing biological oxygen demand, improving textured bottom soils in the presence of organic material, and improving nitrification in soil and water of the grow out systems for shrimps and fishes. They also neutralize acid sulfate resulting from oxidation of pyrites in ponds constructed in mangrove areas.

Drainage from acid sulfate soils can cause extremely low pH in ponds outside the tolerable range of most species.

Fertilization programmes are usually ineffective in ponds with acidic waters and sediments. This reduced response is caused by two factors. The first is a deficiency of carbon in the alkalinity system, which cannot support high rates of photosynthesis by phytoplankton and plants. The second is the increased adsorption of dissolved inorganic phosphorus by sediments. Phosphorus is a key nutrient for phytoplankton growth and its availability limits phytoplankton production. Liming materials may be used to support fertilization programmes or to improve productivity in acidic waters by addressing both these factors.

Lime is generally recommended as a treatment for ponds when total alkalinity and total hardness are below 20 mg/litre. Heavy phytoplankton growth can deplete free $CO_2$, which is required by phytoplankton and aquatic plants for photosynthesis. Bicarbonate ions provide an alternative carbon source for photosynthesis in the absence of free $CO_2$. The increase in total alkalinity resulting from properly applied liming materials is primarily by bicarbonate ions. Liming can therefore favour greater rates of photosynthesis at times when the availability of free $CO_2$ is limited, leading to substantially higher phytoplankton densities. In this situation, phosphorus added to ponds rapidly disappears from solution, part of this being due to absorption by phytoplankton, but most of the phosphorus is removed through reaction with the sediment to form iron and aluminium phosphate compounds. This process is pH dependent. Applying lime to neutralize the acidic muds of ponds to pH 6.5 has been shown to increase soluble phosphorus concentrations, which may enhance phytoplankton productivity.

Agricultural limestone cannot be applied simultaneously with phosphate fertilizers as this will cause phosphorus to precipitate. This is due to the high calcium concentrations in the limestone, which reacts with phosphate to form tricalcium phosphate. It is, therefore, desirable to add liming material well in advance of fertilizers. Liming is not usually considered to be a form of fertilization. However, liming increases the concentration of calcium and/or magnesium, which can be limiting nutrients for phytoplankton at low concentrations. These nutrients are most

likely to be limiting in waters of low total hardness. Lime must be periodically reapplied to remain effective. Ponds treated with approximately 1000 kg/ha agricultural limestone or hydrated lime have been reported to show increased productivity for two to four years. The effective period of an application, as indicated by water hardness, is determined by the rate of water loss to seepage and overflow from ponds. Liming has been reported to be ineffective in a pond with a water retention time of less than three weeks.

4. ***Mode of application***

   Applied as such in the pond bottom before stocking if the lime requirement is experienced. Application in water during culture period in small doses as per pH and alkalinity is also recomended.

5. ***Shelf life and storage***

   Remains stable indefinitly provided it is stored in cool dry place.

6. ***Recommended dosage***

   Lime is applied to the pond bottom at doses of 100-8000 kg/ha or to the water during the rearing period at 10-500 kg/ha depending on the pH.

7. ***Metabolism of the drug in the animal body, retentivity and mode of excretion***

   The chemicals are not known to be consumed.

8. ***Residues in host species***

   No known residual effect.

9. ***Host species safety***

   During normal application no deliterious effects are noticed, provided the unionized ammonia remains below the tolerance limit of the species.

10. ***Environmental safety***

    Being natural products, no environmental hazards are expected.

11. ***Operator safety***

    Liming materials are not hazardous to human beings, but being alkaline can cause skin irritation on handling without precaution.

## 5. Zeolite

1. ***Chemical name***

   Clinoptilolite

   It is a mixture of $SiO_2$ (60-65 %), $Al_2O_3$(18-22 %), $Fe_2O_3$ (2-3 %), CaO (15-18 %) Mg O (2-5 %) $Na_2O$ (1-2%)

   Zeolite is a tecto-silicate mineral and is considered as molecular sieves.

2. ***Mode of action***

Zeolite is applied to shrimp ponds to remove hydrogen sulphide, carbon dioxide and ammonia, as it has a strong capacity to absorb molecules and is used to clean the pond bottom. It reactivates soil, promotes algal growth and absorbs fouling material. The ability of zeolites to chemically reduce ammonia is accomplished by the adsorption of ammonia on to the natural zeolite thereby shifting the $NH_4$ - $NH_3$ equilibrium in the aqueous solution and reducing the potential for $NH_3$ toxicity. When the optimum quantity of zeolites is used, the $NH_4$ level is reduced at a rate highly dependent upon the rate of water movement. However, this activity is very much limited in saline waters.

3. ***Mode of application (including recommended preparations required for applying in aquatic system)***

Diluted in water and sprayed over the pond.

4. ***Shelf life and storage***

Stays indefinitely on storing in a cool dry place.

5. ***Recommended dosage***

Often applied in fish/shrimp ponds at a dose of 100-500 kg/ha to remove ammonia. To reactivate soil and for promoting algal growth in shrimp ponds, a dosage of 250-1000 kg/ha is recommended.

1. ***Metabolism of the drug in the animal body, retentivity and mode of excretion***

The chemicals are not known to be consumed by the animal.

2. ***Residues in host species***

No known residual effect.

3. ***Host species safety***

During normal application no deleterious effects are noticed.

4. ***Environmental safety***

Being a natural product no environmental hazards are expected.

5. ***Operator safety***

Not hazardous to human beings.

## 6. Hypochlorite (Sodium and Calcium)

1. ***Chemical name***

Sodium hypochlorite NaClO

Calcium hypochorite $Ca(OCl)_2$

2. ***Target organism***

A variety of microorganisms such as bacteria, fungi, protozoans and viruses and weed fishes are distroyed by hypochlorites.

3. ***Mode of action***

Hypochlorites act by releasing hypochlorous acid, which is the primary active ingredient. It is a potential germicide. They are particularly effective in acidic conditions. The bactericidal effect of hypochlorite is 10 times greater at pH 6 than at pH 9. At pH 7, 0.1-0.25 ppm hypochlorite solution will kill most organisms with in 15 to 20 seconds.

4. ***Mode of application***

The calculated quantity of sodium hypochlorite and/or calcium hypochlorite can be diluted with water or as such sprayed over water column or on sediment surface after sun set.

5. ***Shelf life and storage***

Shelf life is very short. Sodium hypochlorite will remain stable only at $4^{o}$ C and better to use the entire content on opening the container. Calcium hypochlorite must be maintained in properly sealed polyethelene bags protected from sun light in a cool dry place. The entire bag should be used when opened.

6. ***Recommended dosage***

In shrimp farms, 20-30 ppm of hypochlorite is used for pond disinfection. A dosage of 0.08 ppm is used as prophylaxis in ponds with shrimp. In shrimp ponds, hypochlorites have been traditionally used as piscicides. To kill nematode eggs in ponds, the bottom silt is purged with 50-100 ppm hypochlorite or chlorinated lime is applied. The effectiveness of chlorine is also affected by the amount of organic matter, reduced compounds and turbidity present in the water to be treated. If chlorine is used in water with high organic matter, the rate of application should be higher. The dosage depends on the active ingredient of the residual chlorine.

7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

Hypochlorites are not consumed by the animal or penetrate the tissue. It is an active oxidizing agent and its activity is limited to the animal surface.

8. ***Residues in host species***

No known residual effect.

9. ***Host species safety***

Hypochlorites are too toxic to be used directly on tissues and, therefore, cannot be used for treatment or as prophylaxis.

***10. Environmental safety***

When chlorine is added to pure water it is found predominantly as free available chlorine. This free chlorine is non-toxic at high levels and inactivates pathogenic agents within a short span of time. However, this situation is complicated when it involves water containing ammonia ($NH_3$), as a number of chlorine related compounds called *chloramines* are formed. Although chloramines can kill pathogens, they do so at a much slower rate than free chlorine. They are also responsible for causing eye irritation in humans and other mammals. The degree of mucus membrane and eye irritation is pH dependent; water adjusted to a pH of sea-water (7.8-8.4) will have a predominance of monocholramine ($NH_2Cl$). Fortunately, early research in chlorination techniques found that by continuing to add chlorine to water containing these compounds resulted in a second chemical reaction. This reaction, sometimes referred to as 'break-point', effectively breaks down the troublesome chloramines and results in a predominance of the non-toxic free chlorine. The level of chloramines may reach a point where animals may be injured and burnt. The ratio between free chlorine and chloramin should be 2:1 or better 3:1.

***11. Operator safety***

Inhalation of chlorine emanated from the hypochlorite can be hazardous to human beings.

## 7. Ozone

Ozone is a powerful oxidizing agent with numerous beneficial aquaculture uses. The quality of aquaculture production water can be improved by ozone treatment, which helps improve solid settling and reduces nitrite-nitrogen ($NO_2$-N), color, fine particulate matter, and microbial activity. Ozone shows excellent potential for many aquaculture systems because of its rapid reaction rate, few harmful reaction by-products and molecular oxygen produced as a reaction end product. Use of ozone for aquaculture began in the mid-1970s and was initially focussed on disinfection and color reduction of aquarium system with low fish densities and low feed loadings. Since then, ozone has been used to improve water quality in various types of aquaculture systems– ranging from flow-through raceway systems for salmonids to indoor, recirculating systems. Although ozone has proven effective in the reduction and control of certain water quality characteristics, it is not a one-step water treatment technology. The use of ozone as part of a larger water treatment system maximizes its treatment and cost-effectiveness.

## 8. Benzalkonium chloride

***1. Chemical name***

Benzalkonium chloride

Benzalkonium chloride belongs to quaternary ammonium compounds containing four carbon containing groups and a negatively charged ion such as Bromine or Chlorine.

2. ***Target organism***

   Targeted towards a variety of microorganisms, including *Lagenidium* and *Haliphthoros.* Shrimp farmers use it to reduce the concentration of plankton and dianoflagellates in closed pond systems.

3. ***Mode of action***

   The mode of action includes denaturation of proteins, interference with glycolysis and membrane damage. Most likely site of damage to the cell is the cytoplasmic membrane where the compound alters the vital permeability features of the cell structure.

4. ***Mode of application***

   Diluted in water and sprayed over the pond water.

5. ***Shelf life and storage***

   Stays indefinitely on storing in cool dry place.

6. ***Recommended dosage***

   In shrimp hatcheries and grow out systems it is used in small amounts of 0.1-0.5 ppm.

7. ***Metabolism of the drug in animal body, retentivity and mode of excretion***

   The chemicals are not known to be consumed.

8. ***Residues in host species***

   No known residual effect.

9. ***Host species safety***

   At 10-ppm level, no inhibitory effect on the hatching rate of eggs of *Penaeus monodon* has been noted. But survival rate of hatched nauplii was significantly reduced. If applied in large amounts, the resulting decomposition of organic matter will have an effect on animal health.

10. ***Environmental safety***

    Low toxicity and high solubility in water.

11. ***Operator safety***

    This is not hazardous to human beings, but can cause skin irritation on handling without precaution.

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CHAPTER 5

# Feed Additives*

## Feed Additives

In its broadest sense, a feed additive is any substance added to feed. Legally, the term refers to "any substance, the intended use of which results, or may reasonably be expected to result, directly or indirectly, in its becoming a component, or otherwise affecting the characteristics of any feed." This definition includes any substance used in the production, processing, treatment, packaging, transportation or storage of feed.

If a substance is added to a feed for a specific purpose, it is referred to as a direct additive. Indirect feed additives are those that become part of the feed in trace amounts due to its packaging, storage or other handling. For instance, minute amounts of packaging substances may find their way into feeds during storage.

Why are additives used in feeds?

- To maintain product consistency.
- To improve or maintain nutritional value.
- To maintain palatability and wholesomeness.
- To provide leavening or control acidity/alkalinity.
- To enhance flavor or impart desired color**.**

## 'Natural' and 'artificial' additives

Some additives are manufactured from natural sources such as soybean and corn, which provide lecithin to maintain product consistency, or beet, which provides beet powder used for colouring. Other useful additives are not found in nature and must be man-made. Artificial additives can be produced more economically, with greater purity and more consistent quality than some of their natural counterparts.

## A. Carotenoids (color additive for pigmentation purposes)

A color additive is any dye, pigment or substance that can impart color to the animal, which consumes it.

Carotenoids are a group of fat soluble pigments that absorb light in the 400-500 nm region of the visible spectrum. This physical property provides the characteristic red/yellow colour. The pigments render the red, yellow and orange color to finfish and shellfishes when consumed. Some 600 different carotenoids are known to occur naturally and new carotenoids continue to be identified. Apart from this, carotenoids have roles in reproduction, respiration,

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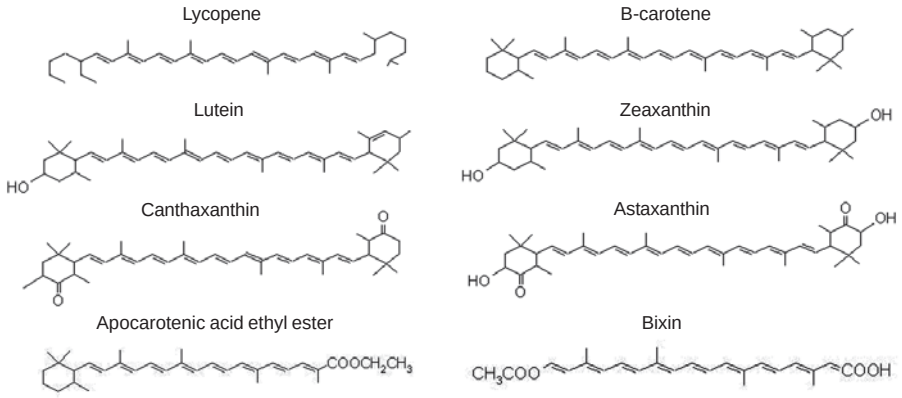
membrane permeability, light absorption, reflection and the efficiency of the immune system. Many animals also use colour to communicate warnings, mating calls, feeding signals and camouflage.

Animals cannot synthesize carotenoids *de novo* and must obtain these from their food. Since carotenoids are only synthesized by plants and modified in animal tissue, fish must obtain them from their diet. Oxygenated carotenoids (xanthophylls) are used by fish and crustaceans. Fish and crustaceans obtain their colors from natural pigments. Within animals carotenoids provide bright coloration and serve as antioxidants and can also be a source of vitamin A activity.

Carotenoids are found widely in nature, the largest groups being the fat soluble carotenes and xanthophylls. The carotenes are found mainly in green plant material and have Alpha ($\alpha$) and Beta ($\beta$) forms. $\beta$-carotene is the precursor for vitamin A and this conversion is carried out universally by most animals. The xanthophylls are synthesized in green material starting early in the summer and maturing to yellow/red pigments. Natural carotenoids are to be found in almost all forms of the living world. Generally, in contrast with the carotenes, the xanthophylls are very well absorbed.

***Chemical name and structure***

The structure is derived from a 40-carbon polyene chain, which could be considered the backbone of the molecule. This chain may be terminated by cyclic end-groups (rings) and may be complemented with oxygen containing functional groups. The hydrocarbon carotenoids are known as carotenes, while the oxygenated derivatives of these hydrocarbons are known as xanthophylls. Beta-carotene, the principal carotenoid in carrots, is a familiar carotene, while lutein, the major yellow pigment of marigold petals, is a common xanthophyll. The distinctive pattern of alternating single and double bonds in the polyene backbone of carotenoids is what allows them to absorb excess energy from other molecules and this accounts for the antioxidant properties. The carotenoids of crustacea are basically C40 structures with $\alpha$- and $\varepsilon$- cyclic end groups. The location of hydroxy - ketone substituents in one or both terminal rings determine the main different




*Chemical structures of some carotenoids*

forms which have been observed in these animals. Many other carotenoids represented by new structural types have been proposed and isolated in several cases.

Three forms of carotenoids occur in biological systems:

a. Esterified - biologically more active
b. Unesterified
c. Protein complexes - Carotenovitellin.

***1. Mode of action***

Powerful intracellular antioxidants protect cells from damage by unstable free radicals and boost the immune system. Though similar in structure, each carotenoid acts on a different type of body tissue.

Pro-vitamin A activity– certain carotenoids, including beta-carotene, alpha– carotene and cryptoxanthin are converted by the body into vitamin A, but only when the body requires it. The role of carotenoids other than as a precursor of Vitamin A in fish is not well defined and mostly speculative.

Recent studies have clearly attributed the significant functional role of carotenoids as powerful intracellular anti-oxidants. Besides, the endogenous defense systems mainly contributed by the anti-oxidant enzymes superoxide dismutase, catalase, peroxidases and small molecules with antioxidant activity– glutathione and hormone melatonin, diet derived anti-oxidants ascorbic acid, alpha– tocopherol and carotenoids are very important for aquatic organisms

***2. Recommended dosage***

Carotenoids are important substances which help to improve color of cultured animals in order to improve market value. Addition of 30-33 ppm astaxanthin also helps in improving the pigmentation in prawns. Crustaceans and polycheates are good sources of carotenoids, carotene and its oxidative derivatives cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin are the preferred carotenoids, though their efficacy markedly differs.

***3. Metabolism of the drugs in animal body, retentivity and mode of excretion***

The metabolism and deposition of carotenoids following absorption from the intestinal tract varies widely from species to species. Some are deposited in the same form, others can be modified. Crustaceans have the ability to convert carotene, lutein and zeaxanthin in algae to the major pigment astaxanthin. In contrast, the carp family has only limited ability to synthesize these major pigments and carotene and lutein are deposited unchanged. However, zeaxanthin can be converted to astaxanthin in skin. This contrasts with salmonids (trout and salmon), which are unable to synthesize astaxanthin and canthaxanthin from other carotenoids. The absorption and accumulation of carotenoids has shown in experiments to vary with chemical structure (*i.e* $\alpha$ or $\beta$ form), solubility and binding form.

β-carotene is only slightly absorbed with very small contribution to colour and conversion to astaxanthin. It appears that α-structure of carotenoids are accumulated better than the β. Metabolism does not seem to be determined by whether it is in free form or ester, but by the chemical structure.

Most green plants contain both α and β-carotene. The algae have a higher amount of α-carotene and alfalfa and grass meal have a preponderance of β-carotene. This would indicate that the blue-green algae meal *(Spirulina)* and algae from ponds would be more efficiently utilized by fish.

Lutein, found in abundance in alfalfa/grass meals and some green algae (*Chlorella*) but not in *Spirulina*, has been scientifically shown when fed in excessive amounts to be a problem. The lutein is first deposited in the pigment cells in the ester form and any excess is then diverted to the white parts giving undesirable yellowing. This shows the importance of having the right balance of pigments in the feed.

As many modern re-circulating and intensive systems for keeping fish/prawn do not allow for sufficient generation of natural food, it is vital to use a high quality food supplement. The diet should not only contain adequate levels of oil, protein, energy, vitamins and minerals but also a constant supply of carotenoids with the correct balance. Unfortunately, reliable sources of carotenoids are relatively expensive compared with the normal fish feed ingredients such as fish meal, meat by-product meals, soya and cereals**.**

## B. Astaxanthin

Astaxanthin is a red pigment occurring naturally in a wide variety of fish and crustaceans including shrimp, crawfish, crabs and lobsters. Astaxanthin not only provides pigmentation to these animals but is also found to be essential for their proper growth and survival. The structure of astaxanthin is very similar to beta-carotene.

### *1. Mode of action*

Astaxanthin binds more tightly to the muscle tissue, forming two bonds compared to one with canthaxanthin. The result is greater intramuscular binding strength. This binding strength is 0.78 mg/g of protein for astaxanthin and 0.47 mg/g for canthaxanthin. Other advantages are greater efficiency in supplementation of flesh color that can better withstand the rigors of processing.

### *2. Mode of application*

Synthetic astaxanthin is the major form currently being used in fish feeds. Crustaceans including Antarctic krill *Euphausia superba* are rich sources. The microalga *Haematococcus pluvialis* is a rich source of natural astaxanthin. The alga is grown in

*Astaxanthin content (mg/kg) of selected natural materials*

| | |
|---|---|
| Crabs | 75 - 1300 |
| Krill | 100 - 130 |
| Shrimp | 25 - 125 |
| Shrimp oil | 1000 |
| Yeast (*Phaffia rhodozygma)* | 30 - 800 |

photobioreactors. Pink yeast *Xanthophyllomyces dendrorhous* also produces astaxanthin in a fermentation process.

*Form and level of astaxanthin in selected finfishes*

| Aquaculture species | Content (mg/kg ) | Astaxanthin free/esterified | Main isomer |
|---|---|---|---|
| Sockeye salmon | 26-37 | free/esterified | 3S,3'S |
| Coho salmon | 9-21 | free/esterified | 3S,3'S |
| Chum salmon | 3-8 | free/esterified | 3S,3'S |
| Chinook salmon | 8-9 | free/esterified | 3S,3'S |
| Pink salmon | 4-6 | free/esterified | 3S,3'S |
| Atlantic salmon | 3-11 | free/esterified | 3S,3'S |

However, sources of astaxanthin for incorporation into the diet of farmed fish are limited and synthetic astaxanthin is traditionally used. In fact, astaxanthin represents the single most expensive component of the diet.

***3. Recommended dosage***

The commercially available astaxanthin is added to the feed for farmed fish in a product form (CAROPHYLL®Pink) in which the pigment stability is increased. The molecule of the astaxanthin used in the product is identical to the molecule present in wild salmon. Astaxanthin is used as a feed additive at no more than 80 mg/kg (72 g/ton) of finished feed to enhance the pink to orange-red color of the flesh of salmonid fish and is an FDA approved flesh color enhancer.

## C. Canthaxanthin

Canthaxanthin is used as a feed additive at no more than 80 ppm to enhance the pink to orange-red color of the flesh of salmonid fish. Canthaxanthin can also be used to give farm-raised salmonids the pinkish colour that consumers expect and is approved by FDA. Yet, astaxanthin will always remain the benchmark in comparison as it accounts for more than 90 percent of the carotenoids found in wild salmonids.

## D. Vitamins

Fish require vitamins in their diet for growth, health and general body functions. Vitamin deficiency has been found to be associated with several pathological signs in fish. Of the water soluble vitamins, vitamin C (ascorbic acid) has attracted the attention of fish health researchers, probably owing to its role in disease resistance and immune response.

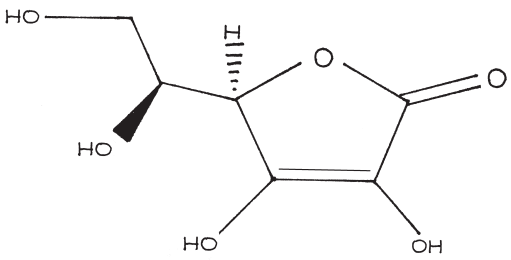
### (a) Vitamin C

***1. Chemical name and structure***

Known as Ascorbic acid, only the L-isomer is the physiologically active form of vitamin C. The R-isomer, which is called erythorbic acid (or occasionally, iso-ascorbic

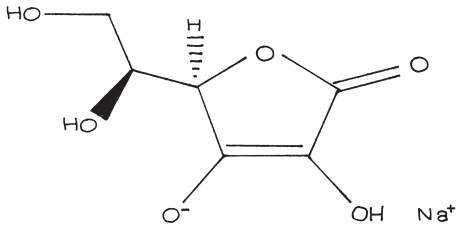
acid) has no vitamin value, although it does function as an *in vitro* antioxidant. Most animal species are able to manufacture L-ascorbic acid in the kidneys from glucose and certain other six-carbon atom chains. A few mammals and birds, notably man and guinea pigs, lack the enzyme L-gulonolactone oxidoreductase, which catalyses the last stage in this synthesis. An exogenous, dietary supply of ascorbic acid is essential for such animals. Ascorbic acid is present in fresh vegetables and fruit. The ingredients used for the manufacture of compound feeds are unlikely to contain any measurable amounts.

*Structure of ascorbic acid: vitamin C*

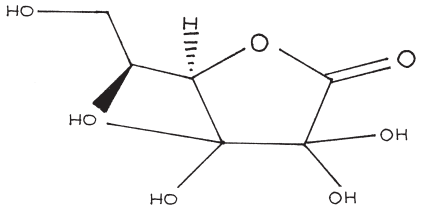



Vitamin C is usually measured in weight units of pure crystalline L-ascorbic acid. There are still occasional references to the International Unit, which is the activity of 50 µg of L-ascorbic acid. Carbon atoms 2 and 3 on the 6-carbon chain are readily oxidized to produce dehydro-L-ascorbic acid that has the same vitamin activity as the original L- ascorbic acid. Sodium ascorbate has 89 of the vitamin value of pure L-ascorbic acid.

*Structure of sodium ascorbate*




*Structure of dehydro-L-ascorbic acid*




### 2. *Target organism*

Diets supplemented with Vitamin C have been reported to give protection against *A. salmonicida* in Atlantic salmon, *Edwardsiella tarda* infections in channel catfish and *Vibrio anguillaram* in rainbow trout. Mega doses of ascorbic acid saved rainbow trouts experimentally exposed to the protozoan parasite, *Ichthyophthirius multifilis.*

### 3. *Mode of action*

Ascorbic acid is transported to all living cells for use in important oxidation/reduction reactions in cell metabolism. It is essential for the formation and maintenance of function of the intercellular substances of skeletal tissues, particularly collagen. It also exerts a stimulant action on defensive mechanisms. According to recent

research, it plays an essential role in transporting iron from plasma to storage sites. It is an important inter-cellular antioxidant and is involved in quenching highly reactive free radicals. In addition to reacting directly with aqueous free radicals, ascorbic acid indirectly affects the balance between oxidative products and antioxidant defense mechanisms. Accordingly, ascorbic acid can donate an electron to tocopherol free radical, regenerating the active tocopherol. The concentration of ascorbic acid in phagocytes in the blood is many times higher than in the erythrocytes and is approximately 150 times the concentration in plasma. These phagocytes use free radicals and other highly reactive oxygen containing molecules to help kill invading pathogens. The antioxidant action of ascorbic acid helps protect these cells from oxidative damage.

***4. Mode of application***

The amount added to feed should reflect the anticipated losses during processing and storage. The amount of crystalline ascorbic acid added to the feed should be increased by a factor of five for pelleting or extrusion, or by 50 % for mixing as dry meal. Ascorbyl poly- or monophosphate is 25 to 35 % of the activity of the crystalline ascorbic acid, and the amount added to feed must be adjusted accordingly. No overage of these salts is necessary to allow for losses in compound production or storage.

Since it has been shown that parasites and infectious diseases seriously affect plasma ascorbate levels, additional exogenous supplies are needed to rebuild normal body pools.

***5. Shelf life and storage***

Crystalline ascorbic acid is relatively stable in air if moisture is completely absent. In the presence of even small amounts of moisture there is rapid oxidation, first to dehydroascorbic acid, and then to other non-vitamin-active products. This irreversible oxidation is accelerated by alkalies and by the presence of metal ions (such as copper). Some oxidative losses occur even during mixing with dry feeds; these are usually between 10-30 %. The amount lost during pelleting or cubing is directly related to the amount of steam used and the temperature of the pelleting chamber. Such losses may amount to 85-90 % of the ascorbic acid added if it is present in the crystalline form. There may be slightly lower losses if the ascorbic acid is coated with ethyl cellulose. It is advisable to add either the ascorbyl polyphosphate or monophosphate salt to pelleted or extruded feed to ensure minimum losses and the presence of the requisite quantity of vitamin C in the finished feed when consumed. Additional ascorbic acid is lost during storage after manufacture when added as the crystalline product. The amount varies according to the temperature and humidity of the store, but is usually about 10 % per month.

The rate of degradation of ascorbic acid in aqueous solutions depends on several factors such as pH (highest stability is shown between 4 and 6), temperature (the higher the temperature the more rapid the loss), the presence of oxygen and the

presence of catalysts such as copper ions. Ascorbic acid in an aqueous solution prepared from ordinary water (which contains dissolved oxygen) drawn from copper pipes will degrade in hours rather than days, even at room temperature. Solutions of ascorbic acid remain stable only if prepared from deionized, de-oxygenated water and bufferted with sodium ascorbate to increase the pH.

***6. Recommended dosage***

Since no vitamin C synthesis occurs in fish, they are dependent on a vitamin supplementation via feed. Fish generally require a minimum of 175 mg/kg feed though there is no specified upper limit. To optimize stability of vitamin C during feed production and storage as well as bio-availability in the fish, the use of vitamin C in phosphorylated form is recommended.

***7. Metabolism of the drugs in animal body, retentivity and mode of excretion***

There is a homeostatic mechanism in the case of mammals which, as the plasma ascorbate level reaches 8 µg/mL, causes the kidneys to withdraw any excess ascorbic acid and excrete it via urine.

Vitamin C is involved in several physiological functions including growth, development, reproduction, wound healing, response to stressors and possibly lipid metabolism through its action on carnitine synthesis. Further, as discussed later, it also plays a significant role in the immune response and resistance to infectious diseases of fish, probably through its antioxidant properties. Vitamin C has no coenzyme functions, unlike other water-soluble vitamins, but acts as a cofactor in many reactions involving hydroxylating enzymes.

*Collagen synthesis:* Collagen is an important component of skin, bone, cartilage and endothelium of blood vessels. Therefore, these tissues will be damaged if the formation of collagen is impaired by insufficient vitamin C levels in the body. The hydroxylation of specific prolyl and lysyl residues of procollagen is catalyzed by hydroxylases and contributes to the stiffness of the collagen triple helix and binds carbohydrates to form intra molecular cross-links which give the structural integrity of the collagen. Ascorbate deficiency also reduces complement activity (the complement component C19 is rich in hydroxyproline and hydroxylysine).

*Catecholamine biosynthesis:* The stress response is primarily controlled by the endocrine system via cortisol and catecholamines whose synthesis depends upon ascorbic acid-dependent hydroxylases. Ascorbic acid requirement is increased by stressful situations. It can compensate for the stress-induced down regulation of the immune system.

**Involvement of vitamin C in other physiological processes**

*Tyrosine metabolism:* The active degradation of tyrosine is made via two oxidases, which are vitamin C-dependent. In turbot, vitamin C deficiency causes hypertyrosinemia and the excretion of tyrosine metabolites.

*Metal ion metabolism:* Vitamin C interacts with several metallic elements of nutritional significance (selenium) and reduces the toxicity of metals such as cadmium, nickel, lead (the elements are transformed into their reduced forms, which are absorbed less and excreted more rapidly). Protects cells from oxidative damage and regenerates vitamin E in its metabolically active form.

*Immune reactions:* Vitamin C affects immune functions in different ways (protection against free radical-mediated protein inactivation associated with the oxidative burst of macrophages, chemotaxis, stimulation of proliferative response, antibody as well as interferon production and helps to maintain the integrity of the immune cells through their protection from oxidation and within the cells. High amount of vitamin C is stored in the immune cells.

**Absorption of vitamin C**

The species that cannot synthesize vitamin C absorbs ascorbic acid by an active transport mechanism which is $Na^+$ dependent. This active uptake of vitamin C seems to be very important at low doses while at high doses, uptake by passive diffusion also occurs. The uptake of vitamin C in cells such as lymphocytes, neutrophils and monocytes involves dehydroascorbic acid because ascorbic acid cannot cross their membrane. Once dehydroascorbic acid is taken up by the cells, it is rapidly reduced to ascorbic acid by an intracellular dehydroascorbic acid reductase.

**Tissue distribution of vitamin C**

Vitamin C is concentrated in many vital organs with active metabolism. The concentration of vitamin C in various tissues is related to the dietary intake of the vitamin. Moreover, some tissues such as brain, thymus and leukocytes accumulate high concentrations. In these tissues, ascorbic acid levels seem to be retained longer in case of dietary vitamin C depletion compared to storage organs such as the liver. The very high levels found in thymus, brain and leukocytes confirm the hypothesis of the importance of ascorbic acid in preserving vital tissues from oxidation processes. Liver and head kidney are important storage organs for vitamin C in fish. The high level found in the head kidney is likely to be related to the presence of lymphopoietic tissues. Trunk kidney and spleen are also able to store a large amount of vitamin C. Ascorbic acid is concentrated at the site of catecholamine formation and it is released with newly synthesized corticosteroids in response to stressors.

With the exception of perhaps two or three species, vitamin C biosynthesis does not occur in fish due to lack of the last enzyme of the biosynthetic pathway: L-gulonolactone oxidase. Therefore, vitamin C must be supplied via feed. Major signs of ascorbate deficiency include reduced growth, scoliosis, lordosis, internal and fin haemorrhage, distorted gill filaments, fin erosion, anorexia and mortality.

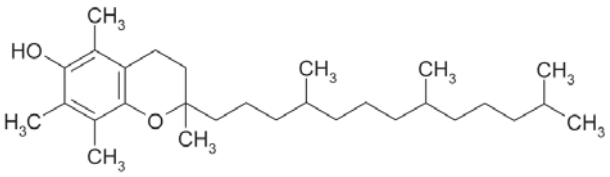
### *8. Host species safety*

There is no reported incidence of toxicity related to the oral administration of vitamin C along with diet.

## (b) Vitamin E

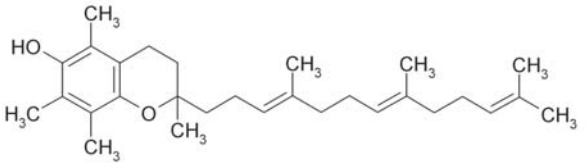
### *1. Chemical name and structure*

Next to ascorbic acid, vitamin E (Tocopherol) has an important role to play in aquaculture. It is also a natural antioxidant. Vitamin E is an essential dietary requirement supplied by a range of components with different activities. There are at least eight different tocopherols with vitamin E activity and all have a 6-chromanol-ring structure and a side chain. Alpha-Tocopherol has the greatest activity and accounts for 70-90 % of the total biologically available vitamin E in most cereal- based mixed feeds. The other tocopherols may be present in greater quantities but they have very low vitamin E activities and are usually discounted in assessments of vitamin E in feeds.




*Structural formula of alpha-tocopherol*

There is also a related family of tocotrienols, which have an unsaturated side chain with three double bonds. Tocotrienols have a lower biological vitamin E value than their related tocopherols.




*Structural formula of alpha-tocotrienol*

*The unit of measurement :* Many tables of values measure vitamin E in International Units. The standard is dl- alpha-tocopheryl acetate.

1 mg dl-alpha-tocopheryl acetate = 1 IU vitamin E

Tocopherols form various stereoisomers that have different activities. This is in addition to the chemical variations between the alpha, beta, gamma and delta forms.

Tocopherols found in feed materials are always a specific stereoisomer, labelled "d" for convenience. This form has greater biological activity than mixtures of isomers (the racemic or "dl" form). Each "d" isomer is 36 % more active than the corresponding "dl" form. In addition to the tocopherols, the tocotrienols have only about 40 % vitamin E activity of the corresponding tocopherol. The biological vitamin E activity is the total of all the activities of the eight homologues, each having eight possible stereoisomers. There is now considerable doubt regarding the biological values of the various isomers.

### *2. Mode of action*

Vitamin E appears to have several different but related functions. One is its role as an inter and intra-cellular antioxidant. It prevents the oxidation of unsaturated lipid materials within cells. If lipid hydroperoxides are allowed to form in the absence of adequate tocopherols, direct cell tissue damage can result. The more active the cell, such as those of skeletal and involuntary muscles, the greater the in-flow of lipids for energy supply and the greater the risk of tissue damage if vitamin E is limiting. This antioxidant property also ensures erythrocyte stability and maintenance of the integrity of capillary blood vessels.

Studies have shown vitamin E to have a regulatory action on the pituitary- midbrain system, promoting the production of hormones that stimulate the output of the thyroid and the adrenal cortex. Though vitamin E is present in all tissues and organs and is essential for the maintenance of the endocrine system, it does not have a specific role in reproduction except in rats as per the present day understanding.

Vitamin E is found in most body tissues; there are no major storage sites although reproductive organs, pituitary and adrenal glands have particularly high concentrations. It is transported in the bloodstream by low-density lipoproteins, and although the amount in transport varies with the time of recent food absorption, it is a reasonably good indicator of status. If muscle tissues have been damaged, such as by myopathy due to vitamin E deficiency, they release their contents of cell enzymes into surrounding tissues and from there into the blood stream. Increased amounts of aspartate aminotransferase (AspAT) or creatine phosphokinase (CPK) indicate tissue damage but are not specifically indicative of low vitamin E status. It appears that increased nutritional supplies of vitamin E can stimulate antibody production.

One of the first functions of vitamin E and selenium to be identified was the maintenance of erythrocyte membrane integrity. Erythrocytes and lymphocytes originate from the same stem cells so it has been suggested that vitamin E and selenium may also be associated with the production of lymphoid cells and thus able to influence immune-response mechanisms. An alternative suggestion is that vitamin E stimulates ubiquinone prostaglandins, which is one of the precursors of the immunoglobulins, or it may limit the biosynthesis of prostaglandins, which tend to inhibit immune responses. Whatever the actual mechanism be, tests have shown that resistance to

disease in a number of species can be improved by increasing the vitamin E input. Some of the early trials were on chickens where immune response, measured both in terms of the amount of immunoglobulins in the blood and by the differences in reaction or mortality between treated birds and controls, was significantly better in birds given extra vitamin E. The first test involved the use of antigenic sheep red blood cells and later tests involved *Brucella abortus* and *E. coli*. Mice and guinea pigs showed similar improvements. Tests with turkeys and pigs, using *E. coli* as the antigen also produced a positive improvement when additional vitamin E was supplied.

Norwegian investigators gave young pigs 300 IU vitamin E daily and then inoculated them with swine dysentery bacterium. Pigs given the extra vitamin E ate more feed, gained more weight and showed much less pronounced clinical signs than the controls without vitamin E. Sheep, dogs and horses have also been used as test animals, with the same effect.

In all these trials it was found that additional vitamin E stimulated IgG synthesis whereas extra selenium promoted increased numbers of IgM-producing cells and thus increased the production of IgM antibody. The dietary levels most effective in these trials were 5 to 10 times the normal dietary supply of vitamin E and 10 to 30 times the dietary requirement of selenium. The stimulation due to selenium was independent of vitamin E supplies but the amounts necessary to produce an effect might be considered toxic and were certainly above the current maximum permitted level.

### *3. Shelf life and storage*

The tocopherols are excellent antioxidants. It, therefore, follows that any condition of feed in which there is active oxygen leads to a reduction in the available tocopherol. Dry grain looses tocopherol very slowly, whereas moist grain loses it very quickly. The supplementary form of vitamin E is alpha-tocopheryl acetate, which is not an antioxidant, and is much more stable to moisture, heat and oxygen. Loss during pelleting is usually around 10-20 %. However, it can be as much as 50 % if water is added to the mix (humidification). The vitamin E acetate is converted to tocopherol during digestion and is absorbed in the small intestine together with emulsified fatty products. All forms of vitamin E are rapidly destroyed by alkalies.

### *4. Recommended dosage*

Where resistance to disease is important, it appears from all these trials that feed supplementation with 150 IU vitamin E per kg feed or more can reduce mortality and minimize the effects of disease challenges in all species. These levels are within the normal range of feed supplies and there is no evidence in any trial that they can produce hypervitaminosis conditions.

## *5. Metabolism of the drugs in the animal body, retentivity and mode of excretion Interactions with other ingredients*

### 1. Selenium

There is a close working relationship between vitamin E and selenium in their functions within tissues. Selenium is an important constituent of the enzyme glutathione peroxidase, which has the important task of removing active peroxides from cells before they oxidize the unsaturated lipids that are protected by the tocopherols. Since vitamin E and selenium have intracellular antioxidant functions, to a small extent they are mutually replaceable, but there are lower limits below which substitution is ineffective.

### 2. Fat

One of the main functions of alpha-tocopherol (vitamin E) is the prevention of the peroxidation of fatty materials within cells. Lipid hydroperoxides can destroy cell and tissues. It is, therefore, logical to conclude that there is a positive correlation between fat input and vitamin E requirements to prevent peroxidation. It also follows that fats already containing peroxides when ingested by the animal need considerably larger amounts of tocopherols to reverse the peroxidation and avoid cell damage. The administration of polyunsaturated fatty acids (PUFAs) increases the vitamin E requirements. Vitamin E was also found to disappear from the rations used, either before or after ingestion, the rate of this disappearance being dependent on the character and rate of oxidation of the accompanying fats. The severity of effect on vitamin E requirements depends first on the degree of unsaturation and also on the length of the carbon chain. C18 and C20 chains are particularly aggressive and two unsaturated links are worse than three. So the order of severity is Arachidic acid (C20-2) >Linoleic acid (C18-2) >Linolenic acid (C18-3). Later research has confirmed that these acids lead to increased vitamin E requirements, but the order of severity, which may vary between species, still remains doubtful.

### 3. Feed attractants

Substances which induce feeding behavior in animals and increase feed intake are termed attractants. Also known as gustatory feeding stimulants they are important as they aid the target organism to detect the scattered feed and thus avoid feed wastage. Almost all the stimulants are nutrients and perceived by chemoreception *viz.* olfaction (smell), gustation (taste) and tactile or sensed by special receptors on contact with very dilute aqueous solutions. Free amino acids and nucleotides with low molecular weights are most important in this category. Generally, a mixture of L-amino acid, glycine – betaine, inosine or inosine-5 phosphate are considered as universal feeding stimulants for fish and crustaceans. Squid, shrimp, clam, mussel and polychaete extracts are known to be excellent natural attractants for prawns and carnivorous fish. Glycine and taurine have been found to be effective as individual amino acids. Trimethyl ammonium hydrochloride (TMAH), a volatile substance is also known to act as a feeding stimulant in prawn.

Betaine is a well known feed chemo attractant in shrimp and fish culture, which complements methionine and choline in the feed digestion, and also boosts efficacy of ionophore anticoccidials. An important role in the activation for the search for feed by aquatic animals is glycine, which is found in high concentrations in various marine invertebrates in the form of glycine-betaine.

In terms of control of feed intake in fishes, olfactory and gustatory stimuli have received the most attention by researchers. Although food may be detected at a distance either visually or chemically by fish, the final decision about whether to swallow or reject potential food material is based on gustation. The feeding behavior of several species of marine and freshwater fishes was found to be mediated by mixtures of chemicals, implying that a number of different chemosensory cells must be stimulated to induce a feeding response. The mixtures that cause the greatest behavioral responses are composed of amino acids, nucleotides and quaternary amines. Among the single compounds that show the highest effectiveness in these mixtures are betaine, glycine, alanine and taurine.

Betaine plus mixtures of amino acids are feeding stimulants for fishes in general. Glycine is particularly a strong attractant for flounder (*Pseudopleuronectes americanus*). Two dipeptides, hypotauryl- 2-carboxyglycine and C-methylimino-diacetic acid were active feeding stimulants for grunt (*Bathystoma rimator*). The turbot (*Scophthalmus maximus*) showed a strong gustatory sensitivity to specific nucleotides such as inosine and inosine 5'-monophosphate.

***Metabolism of the drugs in the animal body, retentivity and mode of excretion***

Most of the amino acids are deaminated by aquatic animals. There is no evidence of residues in fish and crustaceans and is considered safe.

## E. Antimicrobial substances for growth promotion

The use of antibiotics has become an important part of aquaculture where they are used for therapy to treat disease, to control and prevent infection and for growth promotion and production efficiency. The most controversial use of antimicrobial drugs in food animals, except in case of farmed aquatic organisms, involves the administration of antimicrobials for growth promotion or performance enhancement purposes, *e.g*. feed efficiency and digestion enhancement. The matter is complicated by the fact that some drugs are approved for both growth promotion and disease prophylaxis. Even those drugs approved only for growth promotion are believed by many users to be beneficial in disease prophylaxis also.

Growth promoters having antimicrobial property are used in low concentrations in feed to stimulate growth, resulting in increased daily live weight gain and/or feed conversion efficiency. Special care is needed in the use of these compounds since they leave residues that may be potential contaminants for human food supply and the natural ecosystems. Virginiamycin has been used as an animal growth promoter (AGP) in Europe and North America for more than 20 years.

### *1. Mode of action*

It is not precisely known how antimicrobials facilitate growth when fed at low concentrations to animals. Effects may be physiological, nutritional, or metabolic in nature. Improvement in growth performance is probably due to one or more of a variety of mechanisms, including reduction of 'detrimental species' of bacteria, reduction in absolute numbers of microbial organisms (thereby exerting a 'nutrient sparing effect') and reduction in overall infectious disease challenge to the animal. Although gain in weight and feed efficiency may be small on a per-animal basis, the net effect across an entire industry may be quite large. The benefits of growth promoters are reportedly greater under poor hygiene conditions and their current efficacy as disease prophylactics remains controversial now; other means of controlling disease such as biosecurity, vaccination and improved management have been introduced widely into intensive animal husbandry and also finding greater acceptance.

### *2. Mode of application*

Antimicrobials meant for growth stimulation used in feeds include: bacitracin, virginiamycin, rumensin, avotan, payone and carmarin. They are administered in the entire group of fish/shellfish in a tank or pen.

### *3. Environmental safety*

**Transfer of resistance :** One of the drawbacks of administration of antimicrobials meant for growth stimulation is the development of resistance, which is transferable to human pathogens also. This has been demonstrated in bacteria in the water of fish ponds and in marine sediments. Plasmids carrying resistance determinants have also been transferred *in vitro* from fish pathogens to human pathogens, such as *Vibrio cholerae* and *V. parahemolyticus* and to potential human pathogens, including *Escherichia coli*. Furthermore, plasmids carrying multiple anti-microbial resistance determinants have been transferred in simulated natural micro-environments between bacterial pathogens of fish, humans and other animals. Humans who are exposed to aquaculture settings may become infected with bacteria through handling of fish.

The European Union has already recommended a ban on the use of virginiamycin, spiramycin, tylosin phosphate and zinc bacitracin as antibiotics authorized for growth promotion from 1 January 1999. In Canada no antimicrobials are registered for growth promotion purposes in aquaculture.

## F. Enzymes as growth promoters

Feed enzymes have an important role to play in current farming systems. They can increase the digestibility of nutrients, leading to greater efficiency in the production of animal products such as meat and eggs. At the same time they can play a role in minimizing the environmental impact of increased animal production.

Enzymes are naturally occurring proteins that act as biological catalysts. The complex biochemical reactions that form the metabolism of living organisms are regulated by thousands of enzymes, each promoting a specific reaction that takes place countless times every day.

***Mode of Action***

Cereals such as wheat, rye and barley, for example contain long, complex carbohydrate molecules known as non-starch polysaccharides (NSPs) for which animals as such do not produce the necessary digestive enzymes. It is now well recognized that these components are anti-nutritional in behavior. Not only do they increase the viscosity of digests, which means that the animals' own enzymes have a harder time locking onto nutrients and the absorption of these nutrients is reduced, but they also encapsulate nutrients, thus making them unavailable to the animal. The addition of enzymes to animal diets allows the breakdown of these anti-nutritional factors and thus promotes faster and more complete digestion of the feed, leading to improved nutritive value.

Feed enzymes are also able to upgrade sources of vegetable protein (such as soybeans, rapeseed, sunflower seed and legumes) in fish.

The benefits of enzymes include:

- Reduction of feed costs by providing flexibility in feed formulation.
- Improvement of feed efficiency.
- An increase in the dietary content of metabolizable energy improvement in growth rates.
- Enzymes for reducing the phosphorus burden on the environment.

Most (50-80 %) of the phosphorus contained in feedstuffs of plant origin exists as the storage form phytate, or phytic acid. The phytase enzyme is essential for the release of phytate-bound phosphorus. Therefore, sufficient phytase needs to be added to the feed. Phytate also forms complexes with proteins, digestive enzymes and minerals and as such is considered to be an anti-nutritional factor. Phytase frees the phosphorus contained in cereals and oilseeds, and by breaking down the phytate structure also achieves the release of other minerals such as calcium and magnesium, as well as proteins and amino acids, which have become bound to the phytate. Thus, by releasing bound phosphorus in feed ingredients of vegetable origin, phytase makes more phosphorus available for bone growth and reduces the amount excreted into the environment. Use of the enzyme also has the added benefit of helping to conserve natural resources by eliminating the need to supplement feeds with sources of digestible inorganic phosphorus granulate formulation. There are significant differences in stability through feed processing between the various granulate enzyme preparations available in the market.

**Forms of Enzymes**

Product forms have been designed specifically to protect the enzyme during hydrothermal feed processing and subsequently degrade rapidly to release the enzyme in the digestive tract.

Enzyme products are available as liquids and free flowing granulates to suit all feed production systems, allowing maximum flexibility. The use of enzymes as feed additives is restricted in most countries by local regulatory authorities. Applications may, therefore, vary from country to country. Addition of some synthetic enzyme preparations at levels of 0.01, 0.02 and 1.0 % have been known to improve growth of carps.

**(a) Phytase (myo-inositol hexakisphosphate phosphohydrolase)**

Produced by a vaiety of fungi and bacteria which release phytate bound phosphorus from feed. Commercial products are available.

**1.** ***Metabolism of drugs in the animal body, retentivity and mode of excretion***

Enzymes being protein in nature pose no toxicity problem as they get metabolized when they reach the digestive tract of the target species by the proteolytic enzymes present in the digestive system of the animal/fish species. They in fact, when degraded, contribute to the amino acid pool of the animal.

**(b) Bromelain**

**1.** ***Chemical name***

***IUBMB Enzyme Nomenclature EC 3.4.22.32***; Common name: Stem bromelain. Other names: bromelain, pineapple stem bromelain.

***2. Target organism***

Applicable in the case of both fish and prawn, which are fed with prepared diet.

***3. Mode of action***

Broad specificity for cleavage of proteins, but strong preference for Z-Arg-Arg NH Mec amongst small molecule substrates.

Bromelain is a proteolytic enzyme derived from *Bromeliaceae* sp. (Pineapple plant). The proteolytic activity of this enzymes possesses a broad range of substrate specificity and is capable of efficiently hydrolyzing most soluble proteins. During the hydrolytic process, amino acids are liberated by proteolysis but the reaction does not necessarily proceed to completion.

**(c) Papain**

***1. Chemical name***

***IUBMB Enzyme Nomenclature EC 3.4.22.2;*** Common name: papain

Molecular weight of papain is 23 000 and is a single peptide chain of 211 residues folded into two parts that form a cleft. A three-dimensional structure for papain has

been indicated. The molecule has one functional free-SH group. In papain there are seven subsites, each capable of accommodating a single amino acid residue of a peptide substrate.

***2. Mode of action***

Hydrolysis of proteins with broad specificity for peptide bonds, but preference for an amino acid bearing a large hydrophobic side chain at the P2 position. Papain is a sulfhydryl protease from *Carica* papaya latex. A second protease, chymopapain, and a lysozyme have also been isolated from this same source. Since native crystalline papain is quite unreactive until acted upon by mild reducing agents such as cysteine or cyanide, it may exist as a zymogen. Papain will degrade most protein substrates more extensively than the pancreatic proteases. It is also an esterase.

***3. Recommended dosage***

In aquaculture, papain is used at a dosage of 0.2 % of the total diet in fishes and shell fish.

## G. Other Growth Promoters

(a) Chitin and its derivatives such as Chitosan.

(b) Glucosamine

Both chitin and chitosan have a major role in aquaculture. They are non-specific immunostimulants, which are effective on short-term basis.

***1. Chemical name***

2-Amino-2-deoxy – *D* glucosamine

Synonyms and Trade Names:

Chitosan; chitopearl; CTFA04299; deacetylated chitin; Flonac - N; Kytex - H; poliglusam; Sea CureF.

***2. Target organism***

In aquaculture, chitosan has been used as an immunostimulant for protection against bacterial diseases in fish, for controlled release of vaccines and as a diet supplement. Similar dose of chitosan in brook trout has been shown to be immunopotent. It has a high degree of protection against *A. salmonicida* infection for a short duration.

***3. Recommended dosage***

Chitosan was administered to brook trout by injection and immersion and it was found that high levels of protection occurred three days after, but protection was greatly reduced by day 14. Results indicated that 150 mg/kg of chitosan was found effective in shellfish. The injections of chitosan enhanced the function of hemocytes and protection against pathogens. Injection of chitosan though more effective is labor intensive, relatively more time consuming and becomes impractical when shellfishes weigh less than 15g. Thus, another method such as oral administration and immersion

may be adopted. Such methods are non-stressful and allow mass administration to different size groups.

The influence of chitosan on immune response of healthy and cortisol treated rohu (*Labeo rohita*) has been demonstrated. After treatment with chitosan sufficiently, higher responses in almost all assay of non-specific immunity was observed in comparison to their healthy control or cortisol treated counterparts respectively without chitosan treatment.

Protection was offered when feeding was done @ 0.5 gm/100 gm feed for one week.

4. ***Metabolism of the drugs in the animal body, retentivity and mode of excretion***

   Chitosan itself is not a product that occurs in nature. Chitosan is biodegraded within two months in farming soils in the summer.

5. ***Residues in host species***

   Chitosan residues are not likely to be retained in the animal body.

6. ***Operator safety***

   Human exposure to chitosan occurs from the uses listed above. Exposure can occur through multiple sources, with individuals often unaware that they are consuming chitosan. A dieter intentionally ingesting chitosan capsules and unintentionally ingesting chitosan as an excipient in a medication, as an ingredient in low-fat dairy products, and as a preservative sprayed on fruits can easily consume a daily dose of 10 to 20 grams. No epidemiological studies or case reports investigating the association of exposure to chitosan and cancer risk in humans have been identified. Chitosan is a polymer not hydrolyzed by human digestive enzymes, its function as a dietary supplement is dependent on its lack of absorption in the human body.

**Conclusion**

Additives have been used indiscrimintely in aquaculture for growth promotion and increasing production efficiency. However, today food and color additives are also strictly regulated. In the United States, Federal regulations require evidence that each substance is safe at its intended level of use before it may be added to food. All additives are subject to ongoing safety review as scientific understanding and methods of testing continue to improve.

The use of growth promoting antibiotics bacitracin zinc, spiramycin, tylosin phosphate and virginiamycin as feed additives should be phased out. The development of optimized dosing rates and strategies for new antimicrobial products and the use of currently authorized antimicrobial products should be reconsidered in the light of any new scientific evidence. The data on the trends of resistance in bacteria from animals and food of animal origin should be collected along with accurate data on the usage of antimicrobials in animals. Codes of practices for the use of antimicrobials in farm animals should be

developed at the earliest. More research should also be carried out on antimicrobial resistance and alternatives to antimicrobial use in aquatic animals. Till such time only use of safe feed additives like ascorbic acid and vitamin E, which enhance natural immunity, along with enzymes are recommended for growth promotion in aquaculture.

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* * *

CHAPTER - 6

# Anaesthetics and Sedatives for Finfish and Shellfishes*

## Introduction

Anaesthetics are important in fish medicine as they provide immobilization and release from reflex responses to promote survival during severe stress. They also reduce synaptic transmission either by impairing synthesis and release of transmitter substances at central excitatory synapses or reducing responsiveness to the transmitter. They can also suppress the cortisol response to an acute stress such as handling and reduce the mortality if the fish subsequently are exposed without anaesthesia to a second stress. Use of anaesthetics cause a decrease in ammonia and carbon dioxide excretion and presumably in oxygen uptake**.**

Anaesthetics have been used in aquaculture for various practices. Aquaculture research often requires handling of fish/shellfish for weighing, measuring, marking and tagging, breeding and collection of tissue samples for a considerable time period. The use of anaesthetics makes these procedures easier and reduces stress and physical damage to the animal. Anaesthetics are extensively used to sedate and calm fish/shellfish during transportation, particularly during the transport of seed from hatchery to grow out systems and during the transport of brood stock from one place to another. A number of anaesthetic agents have been used to assist in immobilization of brood stock during egg and milt stripping.

Anaesthetics also find use in the treatment of fish diseases. There are times when it becomes necessary to anaesthetize a fish so that it can be examined or handled safely. Procedures such as gill biopsy, injections, dressing of lesions, etc., require anaesthesia.

Presently, anaesthetics are predominantly used in slaughtering and handling processes in fish processing industries to give good quality products. Handling stress and fatigue connected to slaughtering affects the shelf life and organoleptic quality of frozen/chilled fish. The use of anaesthesia delays the setting of rigor mortis, leaving more time for bleeding, cleaning, grading and packing and hence gives a better quality product.

## Purpose of anesthetics in aquaculture

- Handling and transporting fish seed and brooders
- Disease diagnosis
- Medication and vaccination
- Experimental procedures
- Increasing body mass
- To kill or capture

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**Characteristics of an ideal anaesthetic agent**

An agent with the following properties may be considered an ideal anaesthetic agent for finfish/shellfish.

- Can be easily administered
- Induces anaesthesia quickly
- Induces unconsciousness or sedation in a predictable manner related to dose
- Provides immobilization and muscular relaxation
- Provides a rapid, predictable and uncomplicated recovery
- Leaves no tissue residues and requires short withdrawal time
- Uses chemicals which are stable, have a long shelf life and break down rapidly in the environment to safe and physiologically inactive metabolites

**Routes of administration**

The major methods of inducing chemical anaesthesia in fish are:

- Bath immersion
- Intra peritoneal administration
- Dispersal in air
- Oral administration

Immersion is usually accomplished in induction tanks or bags. These should be prepared in advance and filled with water that closely matches the habitat of the fish with regard to pH, temperature, salinity or hardness. Recovery tanks or water should also be prepared in advance to match the habitat water. Some anaesthetic agents are administered by dispersing them in air. For example halothane, a volatile compound is administered this way. The anaesthetic concentrations are prepared by soaking pieces of cotton wool in a measured quantity of anaesthetic and subsequently covering them with a glass bell. It is considered that the required concentration of the anaesthetic in air under the glass bell is obtained when the pieces of cotton wool gets dry.

In the case of new species, it is a good practice to anaesthetize a few individuals first, to observe induction and recovery closely before anaesthetizing larger numbers. It is advisable to start with a relatively induction dose. Regardless of the drug being used, all possible steps should be taken to reduce additional stressors on the fish being anaesthetized. Responses to stress will increase the dose needed for effective anaesthesia and will therefore reduce the margin of safety.

**Stages of anaesthesia**

**There are several stages of anaesthesia, which are briefly mentioned below:**

1. **Light sedation:** The fish appears normal. There is a slight loss of reactivity to external stimuli and a slight decrease in opercular rate.
2. **Deep sedation:** The fish maintains normal equilibrium. There is a total loss of reactivity except to strong external stimuli and the opercular rate is slightly decreased.

3. **Partial loss of equilibrium:** The swimming becomes erratic, opercular rate is increased, and reactivity only to strong tactile and vibration stimuli. There is a partial loss of muscle tone.
4. **Total loss of equilibrium:** There is a total loss of muscle tone, equilibrium and spinal reflexes. The opercular rate becomes slow and regular.
5. **Loss of reflex reactivity:** There is a total loss of reactivity; opercular movement is slowed and irregular and the heart rate becomes very slow.
6. **Medullary collapse:** This is the final stage where there is a total loss of opercular movement followed by cardiac arrest.

The stages of anaesthesia reached depend on several factors such as dosage, period of immersion, temperature, fish species and the condition of the fish.

**Monitoring of anaesthesia**

Operculum movement is the best indicator for the stages of anesthesia during induction and recovery.

**Mode of action**

Anaesthetic agents are drugs that reversibly impair neuronal function. Generally, anaesthetics impair neuronal function of the central nervous system (CNS). At therapeutic concentrations, their most prominent effect is the induction of unconsciousness. They interact with hydrophobic components of the neuronal membranes causing them to swell and allow an increase in the fluidity of membrane phospholipids. The neural membranes most susceptible to the physical changes caused by anaesthetics are those of central synapses. Anaesthetics reduce synaptic transmission either by impairing synthesis and release of transmitter substances at central excitatory synapses or by an action on the post-synaptic membrane, reducing responsiveness to the transmitter.

**Factors influencing action of anaesthetics**

**Biological factors**

***Species***: Differences in body design and habit; gill area to body weight ratio
***Strain/genetic variance***: Physiological variability
***Size/weight***: Differences in enzymes
***Sex/sexual maturity***: Change in metabolic rate; lipid content
***Lipid content***: Especially in gonads; lipophilic drugs
***Body condition***: Oily fish/older fish
***Disease status***: Exhausted animals, post-spawners
***Stress***: Weakened/exhausted animals

**Environmental factors**

***Temperature***: $Q_{10}$ as in all ectotherms (poikilotherms)
***pH***: pKa effects and ionization of molecules

***Salinity***: buffering effects

***Mineral content of environment***: Calcium antagonism

**Anaesthetics and legislations**

Developed countries like Europe, North America, Australia and New Zealand have legislation for the use of anaesthetics in the following categories:

1. Safe operator practice for use and safe storage of drug and chemicals.
2. Safe use of drugs with animals intended for human consumption.
3. Potential release of drug into the environment.
4. Animal welfare and experimentation.
5. Safety legislation concerning electric fishing and similar electrical apparatus.

## 1. MS-222

**Synonyms**

Tricaine mesilate, Tricaine methanesulfonate, m-aminobenzoic acid ethyl ester methane sulfonate.

**Chemical name/formula:** 2-methyl 4-sulpholnyl aminobenzoate: $C_9H_{11}NO_2CH_4SO_4$ $[H_2NC_6H_4CO_2C_2H_5.CH_3SO_3H]$

It is an isomer of benzocaine with the amino group in the meta position on the benzene ring rather than the para position. The presence of the methanesulfonate group allows MS-222 to dissolve in water unlike benzocaine, which must first be dissolved in an organic solvent prior to placing in water. It is also lipid soluble. Solutions of tricaine are acidic and should be buffered to pH 7.0 to 7.5 with $NaHCO_3$ or Tris buffer.

MS-222 is rapidly absorbed through the gills. Its mode of action is by preventing the generation and conduction of nerve impulses. It is readily metabolized by liver, kidneys, blood and muscles. The primary metabolites are the acetyl conjugates of ethyl m-aminobenzoate (non-polar) and m-benzoic acid (polar). MS-222 and its non-polar metabolites are excreted through the gills whereas polar metabolites are excreted through the kidneys.

**Applications:** MS-222 is the only anaesthetic agent licensed for use with fish intended for human consumption and ornamental fish. It is used worldwide. It is used to sedate fish for handling and transportation, to anaesthetize fish for vaccination and minor surgery. To perform major surgery and for euthanasia, a higher dose of MS-222 is used. It is also used for toxicity studies.

**Precautions**

- The fish should be kept starved for at least 12 to 24 hours before anaesthetizing.
- MS-222 is a hypoxic agent, so the anaesthesia vessel should be vigorously aerated.
- It is advisable to monitor the effect on a few test fish and to continue observation for 12 to 24 hours after anaesthesia to ensure full recovery of the fish.

- Wearing gloves is recommended to prevent systemic absorption as it causes occupational hazard (retinopathy).
- 21 days withdrawal period for human consumption of MS-222 anaesthetized fish.

**Health status:** It reduces $NH_3$ excretion but not the $CO_2$ excretion; therefore water pH drops. It causes chemical stress and has disadvantageous effect on egg and sperm. MS-222 causes hypocholesterimia, increases ACTH, urea nitrogen, blood glucose and lactate production. It decreases $K^+$, $Mg^{++}$ and Hb and intrarenal ascorbate concentration.

**Dosage:**

| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| MS-222 | Salmonids & tropical fish | 50-100 | <3 minutes | <10 minutes |
| | Halibut | 250 | – | – |
| | Red drum | 80 | – | – |
| | Carp | 40 | – | – |
| | Cod | 75 | – | – |
| | *Pinctada radiata* | 1 ppt | – | – |
| | *Penaeus indicus* | 150 | – | – |
| | *Gammarus pulex* | 0.5-1.0g/l | – | – |

## 2. Benzocaine

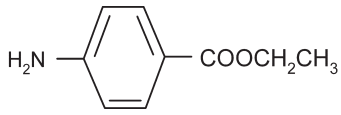
**Synonyms:** Ethyl aminobenzene, p-aminobenzoic acid ethyl ester.

**Chemical name/formula/structure:** Ethyl-4-aminobenzoate: $C_9H_{11}NO_2$ $[H_2NC_6H_4CO_2C_2H_5]$

It is supplied as a water insoluble, white crystalline powder. This powder requires reconstitution in ethanol or ether prior to its addition to water. Its solubility and efficacy in fresh water does not appear to be affected by variations in water hardness and pH, but solubility increases with temperature.

The dosage varies from 50 to 500 ppm depending on the species. The recovery time is prolonged due to its fat soluble property. It varies according to the amount of stored body fat.

**Application:** It has widespread use in anaesthetizing fish and shellfish. It is used for sedation and preferred in experimental procedures. Very low doses of 0.06 -0.2 mg/L are used for transportation of salmonids.



**Precautions**: If a stock solution is to be made in advance, then it should be buffered and stored in dark containers to prevent inactivation. Strong aeration of anaesthetic water is necessary.

**Health status:** Due to the hypoxic effect of this drug, gill ventilation is reduced and subsequently results in depression of medullary respiratory centers. Drug accumulates in adult fish and gravid females.

**Dosage:**

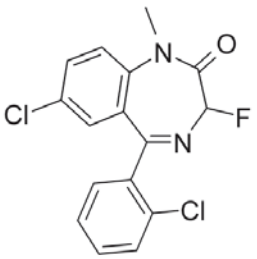
| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Benzocaine | Trout & salmon<br>Striped bass<br>Carp<br>Cod | 33-50<br>55-80<br>50-100<br>40 | 2-4 minutes | Prolonged due to fat solubility |

### 3. Diazepam

**Chemical name and structure:** Benzodiazepine

This is a benzodiazepine group of drugs. They are weak bases and are mostly highly lipid soluble. This drug is very effective for inducing sleep and at relieving psychic and somatic symptoms of anxiety. It also depresses the neural activity. It is a safe drug. The half-life of diazepam is 10-40 hrs. Diazepam undergoes N- 1- demethylation to oxazepam that has central nerve system depressant actions similar to diazepam.

**Application:** It is used for sedation purpose. It is suitable for handling, sorting and transportation of fishes.




**Health status:** It has been proved that Ht, Hb, glucose, Cl-, $Mg^{++}$, leucocyte, neutrophils numbers, lymphocyte (%), activity of AST, ALT, ALP in serum total protein, globulin level and phagocytic activity of neutrophils were more stabilized in fish treated with Diazepam. Long-term exposure in pond experiment with feed for carps showed increase in body mass of fish .

**Dosage**

For common carp and Indian major carps the dosage is 0.5-1.0 gm/kg.

### 4. Quinaldine & Quinaldine Sulfate

**Synonyms:** Quinaldine is known as methylquinoline and quinaldine sulfate is known as quinate.

**Chemical name/formula:** 2-methyl quinolone, $C_{10}H_9N$; 2-methyl quinolone sulphate, $C_{10}H_9N.H_2SO_4$. Quinaldine is slightly soluble in water, but insoluble in acetone and ethanol.

Quinaldine sulfate is soluble in water. In solution, it is acidic and requires buffering with sodium bicarbonate. Quinaldine sulfate is not metabolized by fish and is excreted mainly at the gill level; also excreted by way of kidney and bile.

**Application**: It has common usage and mainly employed for transportation and handling of fishes.

**Precautions**: Care should be taken during its use since it is suspected to be carcinogenic. Stock solution should be protected from exposure to light and air.

**Dosage:**

| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Quinaldine sulphate | Salmonids | 15-40 | 2-5 minutes | 2-60 minutes |
| | Channel catfish | 30-70 | | |
| | Largemouth bass | 15-70 | | |
| | Red drum | 20-30 | | |

## 5. Metomidate

**Synonyms:** 1- (1-phenylethyl)-1H-imidazole-5-carboxylic acid methyl ester.

**Chemical name:** di-1-(1-phenylethyl)-5-(methoxycarbonyl)imidazole hydrochloride.

It is a rapidly acting, water-soluble, non-barbiturate, imidazole-based hypnotic agent. Induction of anaesthesia is very rapid (<5minutes). Recovery time depends on exposure time.

**Applications**: It is not widely used due to its high cost. This is not recommended for use in surgical procedures due to its lack of analgesic properties. It is only used for sedation and also for experimental purposes. It has been reported to be an effective agent to calm broodstocks of Atlantic salmon at low temperature.

**Health status:** Some species of fish turn black after anaesthesia due to the metabolic blockage of cortisol production through the suppression of β hydroxylation of cholesterol. It also inhibits the production of ACTH, which is directly linked to MSH synthesis.

**Dosage:**

| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Metomidate | Salmon | 5 | <3 | 8-20 |
| | Cod | 5-20 | minutes | minutes |
| | Halibut | 20-30 | | |
| | Red drum | 7 | | |
| | Catfish | 1.0-2.5 | | |
| | Tropical fish | 2.5-5.0 | | |

## 6. Etomidate

**Synonyms:** Hypnomidate**,** amidate.

**Chemical name:** An imidazole derivative.

**Applications:** Sedation during transportation.

**Dosage:**

| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Etomidate | Salmonids | 1.0 | 3-5 minutes | 5-20 minutes |
| | Striped bass | 1.0 | - | - |
| | Channel catfish | 3.0 | 15 minutes | |
| | Aquarium fish | 2-4 | 1.5 minutes | Within 40 minutes |
| | *Sciaenops ocellatus* | 0.4-1.8 | Long | Long |

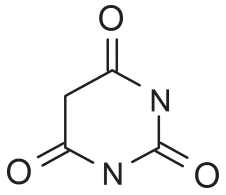
## 7. Barbital Sodium

**Synonyms:** Sodium amilobarbitone.

**Chemical name:** 5-ethyl-5-isoamylbarbituric acid sodium salt.

**Chemical formula:** $C_{11}H_{17}N_2O_3Na$

**Chemical structure:** Barbituric acid



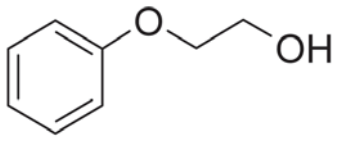

Sodium salt of barbituric acid is called barbital sodium. This is a barbiturate, normally of intermediate duration in mammals, which is easily available as a very stable injectable solution. It is effective in rainbow trout and brown trout at higher intraperitoneal doses of 48-72 mg/kg. Anaesthesia is very lengthy, extending from 6-24 hours depending on dose. The main complications are that recovery is very prolonged with persistent anoxia.

**Applications:** Transportation

**Dosage:**

| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Barbital sodium | | 50 mg/kg fish | - | - |

### 8. 2 - Phenoxyethanol




**Synonyms:** Phenoxethol, chlorophenoxethol

**Chemical name:** 1-hydroxy-2-phenoxyethane

**Chemical formula:** $C_8H_{10}O_2$ [$C_6H_5OCH_2CH_2OH$]

**Chemical structure:**

It occurs as an oily liquid having both bactericidal and fungicidal properties. Induction time and recovery time are concentration dependent.

**Application:** It has minor use globally. It is used to induce light anaesthesia for experimental procedures. It is also suitable for transportation of ornamental fish up to 48 hrs.

**Health status:** It is reported to have a narrow margin of safety and causes hyperactivity during induction or recovery. It significantly reduces the $CO_2$ and $NH_3$ excretion.

**Dosage:**

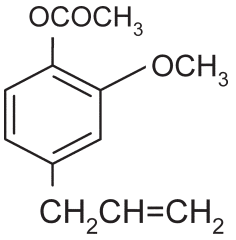
| Drug | Species | Dosage (ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| 2-Phenoxy-ethanol | Salmon | 0.25-0.5 | 2-4 minutes | 3-5 minutes |
| | Cod | 0.1-0.5 | | |
| | Goldfish | 0.1-0.2 | | |
| | *Haliotis midae* | 0.05-0.3ml/100ml | | |
| | Clams | 1 % | | |

### 9. Clove oil

Clove oil is composed of phenols such as eugenol, eugenol acetate and kariofilen-5. The primary constituent of eugenol is chemically 4-allyl-2-methoxy phenol. It makes up to 90-95 % of clove oil.

Clove oil originates from the flowers, flower stalks and leaves of the clove tree *Eugenia aromatica*. It is a distilled product. It is not completely water-soluble and should be mixed with ethanol or acetone at a standard rate of 1:10 (*i.e.,* 100 ml eugenol in 1L of solvent).

Concentrations between 25-100 ppm have proved to be effective in most of the fishes.



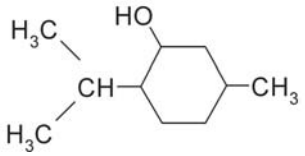

**Applications**: Used for handling and transportation of fishes. Due to its eco-friendly nature, clove oil can be used for wild capture of fish, especially coral reef food fish and ornamental fish.

**Dosage:**

| Drug | Species | Dosage(ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Clove oil | Rabbit fish<br>Milkfish<br>Striped mullet | 50-100<br>50-100<br>50-100 | 2 minutes or less | <5 minutes prolonged in some cases |

## 10. Menthol

Menthol is a naturally occurring monocyclic terpene found in the oil of the mint tree, *Mentha arvensis*.




**Chemical name** : Menthol

**Chemical formula** : $C_{10}H_{20}O$

**Application:** Transportation and handling of shellfishes

**Dosage:**

| Drug | Species | Dosage(ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Menthol | | 1-2 g/100ml | - | - |

## 11. Carbon dioxide

**Chemical formula**: $CO_2$

Anaesthetic properties of carbon dioxide are well documented. It is extremely soluble in water (880 $cm^3 l^{-1}$) and the technique simply involves bubbling of the gas into the medium.

It is an effective anaesthetic in fish but has only occasional use as a sedative for transportation.

**Health Status:** Partial pressure of oxygen (400-480 mg Hg) is needed to maintain high level activity in carp**.** Hyper activity and stress can be reduced by buffering the water with sodium bicarbonate. Carbondioxide anaesthesia has more disruptive effect on the carp ECG than that of MS-222.

**Application:** For transportation of carps, its effects are comparable with those of MS-222 and quinaldine. Currently, widely used in the salmon farming industry, where it is used for bulk "bleeding" of animals to be harvested for smoking.

**Dosage**:

| Drug | Species | Dosage(ppm) | Induction time | Recovery time |
|---|---|---|---|---|
| Carbon dioxide | *Oncorhynchus mykiss (fingerling)* | 120-150 mg/l | - | - |
| | (adult) | 200-250 mg/l | | |
| | Cyprinus carpio | 95-115mmHg | | |

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* * *

## ANNEXURE 1

**Commercially available anaesthetics and antistress compound**

**Pure chemicals:** Sigma and E-Merck.

**Commercial preparations:** MS-222

| | |
|---|---|
| **a. Tricane-S-tm**<br>**b. Meta-tm** Fort Dodge Laboratories<br>**c. Finquel** | USA, Alpharma Animal health Ltd. U.K<br>(Email:askanimalhealth.uk@alpharma.com) |

Commercial preparation of Aqui - S is available from New Zealand.

Antistress compound for fish seed transportation: ‘‘Cifelostress’’ is available from:

Aqua-Vet Laboratories,
16, Mahabir Tower, Ground Floor Main Road
Ranchi– 834 001, India
Phone: 651-312997, 308862 Fax: 651-317368
E-mail: avl_ranchi@hotmail.com
Website: www.aquavetlab.com

CHAPTER 7

# Application of Hormones in Aquaculture*

## Introduction

Application of hormones in aquaculture dates back to 1930s, when attempts were made by Houssay in Argentina to induce fish to breed using pituitary hormones. In 1934, von Ihering and his co-workers from Brazil developed the hypophysation technique for induced breeding of fish. Since then hypophysation has become a common tool, the world over, for production of fish seed. In 1977, the Chinese demonstrated the usefulness of luteinizing hormone release hormone (LH-RH) in induced breeding of fishes. This was observed in the anti-gonadotropic action of dopamine and in 1988 they developed a preparation using salmon LH-RH and dopamine antagonist (like primozide or domperidone) for commercial breeding of cultivable fish species. Since then a number of commercial preparations like Ovaprim, Ovatide, Ovaplat, etc., containing super active analogs of gonadotropin release hormone (GnRH) and a dopamine antagonist are available in the market for fish seed production throughout the world. Similarly, gonadal hormones are also finding their application in aquaculture. In 1953, Tokio-O-Yamamoto demonstrated the possibilities of changing sex in fish with gonadal hormone treatment. Successful manipulation of the sex and production of an all-male brood in tilapia using gonadal hormone has been achieved. Similarly, in salmon and common carp, sex reversal with gonadal hormone treatment has also been accomplished. Now, gonadal hormones are used in aquaculture to manipulate sex, to control unwanted reproduction, and also to improve growth in tilapia in a large number of countries. In spite of the large-scale use of these gonadal hormones in aquaculture for the last 25 years, there is still a ray of suspicion about the consumer safety of fishes treated with hormones.

An attempt is made in this paper to provide basic information on the commonly used hormones and their acceptability in different countries.

## Hormones used in aquaculture

### 1. Growth Hormone (GH)/Somatotropin

| | | |
|---|---|---|
| 1. Hormone name | : | Growth Hormone/Somatotropin is a protein with 191 aminoacids in the peptide chain. M.Wt. 22 000-26 000. |
| 2. Functional component | : | The entire hormone. |
| 3. Mode of action | : | It enhances growth by stimulating appetite and improving conversion efficiency. |

* ***G P Sathyanarayana Rao***, ***K Narayanan*** *and* ***K V Mohire****, University of Agricultural Sciences, Hebbal Campus, Bangalore– 560 024, Karnataka.*

| | | | |
|---|---|---|---|
| 4. | Mode of application | : | Through intra peritoneal or intramuscular route |
| 5. | Side effects, if any | : | Not known. In higher forms it is known to induce diabetes |
| 6. | Deleterious effects in the environment, if any | : | Not known |
| 7. | Recommended preparations required for applying in aquaculture | : | Injectables are to be prepared |
| 8. | Metabolism of the hormone in the animal body and mode of excretion | : | Readily metabolized and excreted |
| 9. | Residues in animals and environment | : | Not known |
| 10. | Target organism | : | Fish |
| 11. | Shelf-life | : | Can be lyophilized and stored at ambient temperature |
| 12. | Uses | : | Used to enhance growth in fish |
| 13. | Recommended dosage | : | 5-30 µg/g. B.Wt. |

**2. Gonadotropin Release Hormone (GnRH)**

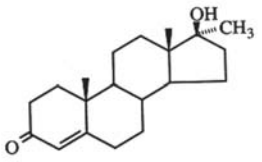
| | | | |
|---|---|---|---|
| 1. | Hormone name | : | Gonadotropin Release Hormone (GnRH); it is a decapeptide; M.Wt. 1212.3 |
| 2. | Functional component | : | The entire hormone |
| 3. | Mode of action | : | It stimulates the synthesis and release of gonadotropins from the pituitary, which precipitates spawning |
| 4. | Mode of application | : | Administration through intramuscular route |
| 5. | Side effects, if any | : | Not known |
| 6. | Deleterious effects in the environment, if any | : | Not known |
| 7. | Recommended preparations required for applying in aquaculture | : | GnRH in combination with a dopamine antagonist like primozide or domperidone shall have to be prepared in a solution for administration |
| 8. | Metabolism of the hormone in the animal body and mode of excretion | : | Readily metabolized and excreted through urine |

| | | |
|---|---|---|
| 9. Residues in animals and environment | : | Not known |
| 10. Target organism | : | Fish |
| 11. Shelf life | : | Can be lyophilized and stored at ambient temperature |
| 12. Uses | : | In the induced breeding of fishes for seed production |
| 13. Recommended dosage | : | GnRH (5-10 μg/kg) in combination with some dopamine antagonist like primozide or domperidone (10 mg/kg). |

**3. Human Chorionic Gonadotropin (HCG)/Chorionic Gonadotropin(CG)**

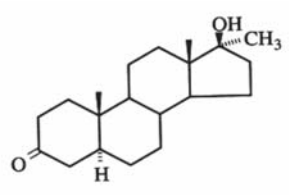
| | | |
|---|---|---|
| 1. Hormone name | : | Human Chorionic Gonadotropin (HCG)/ Chorionic Gonadotropin(CG). It is a glycoprotein hormone with two peptide chains: α-92, β-145 aminoacids. M.Wt. 3 86 000 |
| 2. Functional component | : | The entire hormone |
| 3. Mode of action | : | It stimulates the gonads and induces ovulation and spawning in mature fish |
| 4. Mode of application | : | Through intra peritoneal or intramuscular route |
| 5. Side effects in animals, if any | : | Not known |
| 6. Deleterious effects in the environment, if any | : | Not known |
| 7. Recommended preparations required for applying in aquaculture | : | To be dissolved in distilled water for injection |
| 8. Metabolism of the hormone in the animal body and mode of excretion | : | Readily metabolized and excreted through urine |
| 9. Residues in animals and environment | : | Not known |
| 10. Target organism | : | Fish |
| 11. Shelf-life | : | Can be lyophilized and stored at ambient temperature |
| 12. Uses | : | In the induced breeding of fishes for seed production |
| 13. Recommended dosage | : | 100-500 I.U./B.Wt |

**4. 17α-Methyltestosterone (MT)**



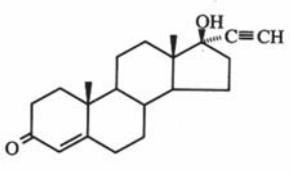

1. Hormone name, formula and structure : 17α-methyltestosterone; $C_{20}H_{30}O_2$; M.Wt. 302.5
2. Functional component : Perhydrocyclopentane-phenanthrene nucleus
3. Mode of action : The hormone, when administered:
   i) At low dose (25-100 ppm), stimulates the undifferentiated gonad to differentiate itself into testis.
   ii) At high dose (100-1000 ppm) destroys the germ cells and the gonadotropic cells and induces sterility.
   iii) At very low dose (1-10 ppm) improves the appetite and food conversion efficiency.
4. Mode of application : The hormone is applied through feed or by adding the hormone to the medium.
5. Side effects in animals, if any : Hepato - toxicity is observed during treatment.
6. Deleterious effects in the environment, if any : Not known. It is photosensitive and it is also reported that there are many microbes in nature, which can metabolize it.
7. Recommended preparations required for applying in aquaculture : Incorporation of hormone into the feed.
8. Metabolism of the hormone in the animal body and mode of excretion : Like the natural hormone testosterone, this hormone also gets quickly metabolized and excreted through urine.
9. Residues in animals and environment : The hormone, like the natural hormone, gets metabolized and more than 90% of it is eliminated from the body within 24 hours.
10. Target organism : Fish and shell fish.
11. Shelf-life : 90 days from the date of incorporation of the hormone into the feed. The hormone is photosensitive and it should not be exposed to direct sunlight.
12. Uses : Used to produce all-male/sterile seed of fish and shellfish and improve growth.
13. Recommended dosage : If administered through diet for the production of : i) all-male seed 25-100 ppm, ii) sterile seed 100-1000 ppm and 1-10 ppm for improving growth. If administered through immersion 50-500µg/L.

**5. 17α-Methyldihydrotestosterone (MDT)**



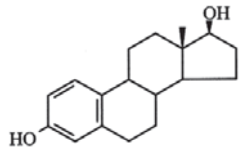

| | | | |
|---|---|---|---|
| 1. | Hormone name, formula and structure | : | 17α-methyldihydrotestosterone; $C_{20}H_{32}O_2$; M.Wt. 304.5 |
| 2. | Functional component | : | Perhydrocyclopentane-phenanthrene nucleus |
| 3. | Mode of action | : | The hormone, when administered at low dose (25 to 100 ppm) stimulates the undifferentiated gonad to differentiate itself into testis. |
| 4. | Mode of application | : | The hormone is applied through feed or by adding the hormone to the medium. |
| 5. | Side effects in animals, if any | : | Hepato - toxicity is observed during treatment. |
| 6. | Deleterious effects in the environment, if any | : | Not known. It is photosensitive and it is also reported to get metabolized by any microbes in nature. |
| 7. | Recommended preparations required for applying in aquaculture | : | Incorporation of hormone into the feed. |
| 8. | Metabolism of the hormone in the animal body and mode of excretion | : | Like the natural hormone testosterone, this hormone gets quickly metabolized and excreted through urine. |
| 9. | Residues in animals and environment | : | The hormone, like the natural hormone, gets metabolized and more than 90 % of it is eliminated from the body within 24 hours. |
| 10. | Target organism | : | Fish and shell fish. |
| 11. | Shelf-life | : | 90 days from the date of incorporation of the hormone into the feed. The hormone is photosensitive and it should not be exposed to direct sunlight. |
| 12. | Uses | : | Used to produce all-male seed of fish. |
| 13. | Recommended dosage | : | It is administered through diet for the production of all-male seed @ 25-100 ppm. |

**6. 17α-Ethynyltestosterone (ET)**




1. Hormone name, formula and structure : 17α-Ethynyltestosterone; $C_{21}H_{28}O_2$; M.Wt. 312.5
2. Functional component : Perhydrocyclopentane-phenanthrene nucleus
3. Mode of action : The hormone, when administered at low dose (25 to 100 ppm), stimulates the undifferentiated gonad to differentiate itself into the testis.
4. Mode of application : The hormone is applied through feed or by adding the hormone to the medium.
5. Side effects in animals, if any : Hepato- toxicity is observed during treatment.
6. Deleterious effects in the animal body : Not known. It is photosensitive and many microbes in nature can metabolize it.
7. Recommended preparations required for applying in aquaculture : Incorporation of hormone into the feed.
8. Metabolism of the hormone in the animal body and mode of excretion : Like the natural hormone testosterone, this hormone also gets quickly metabolized and excreted.
9. Residues in animals and environment : The hormone, like the natural hormone, gets metabolized and eliminated from the body.
10. Target organism : Fish and shell fish
11. Shelf life : 90 days from the date of incorporation of the hormone into the feed. The hormone is photosensitive and it should not be exposed to direct sunlight.
12. Uses : Used to produce all-male seed of fish.
13. Recommended dosage : It is administered through diet for the production of all-male seed @ 25-100 ppm.

**7. 17β-0estradiol ($E_2$)**

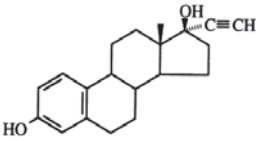



1. Hormone name, formula and structure : 17β-0estradiol; $C_{18}H_{24}O_2$ ; M.Wt. 272.4
2. Functional component : The aromatic ring A
3. Mode of action : The hormone, when administered stimulates the undifferentiated gonad to differentiate itself into an ovary.

4. Mode of application : Through feed or by immersion in the medium containing the hormone.
5. Side effects, if any : Not known
6. Deleterious effects in the environment, if any : Not known. It is photosensitive and it is also reported to get metabolized by many microbes in nature.
7. Recommended preparations required for applying in aquaculture : Incorporation of hormone into feed.
8. Metabolism of the hormone in the animal body and mode of excretion : It is a natural hormone and gets quickly metabolized and excreted.
9. Residues in animals and environment : The hormone gets metabolized and more than 90 % of it is eliminated from the body within 24 hours.
10. Target organism : Fish and shell-fish.
11. Shelf-life : 90 days from the date of incorporation of the hormone into the feed. The hormone is photosensitive.
12. Uses : Used to produce all-female seed of fish.
13. Recommended dosage : 20-120 ppm through diet. If it is through immersion @ 75-400mg/L.

**8. 17α-Ethinyloestradiol**

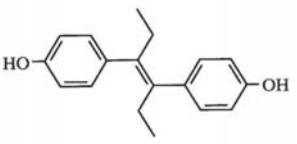
1. Hormone name, formula and structure : 17α-Ethinyloestradiol ($C_{20}H_{24}O_2$); M.Wt. 296.4




2. Functional component : The aromatic ring A
3. Mode of action : The hormone, when administered stimulates the undifferentiated gonad to differentiate itself into an ovary.
4. Mode of application : Through feed or by immersion in the medium containing the hormone.
5. Side effects in animals if any : Not known

| | | | |
|---|---|---|---|
| 6. | Deleterious effects in the environment, if any | : | Not known. It is photosensitive and it is also reported to get metabolized by many microbes in nature. |
| 7. | Recommended preparations required for applying in aquaculture | : | Incorporation of hormone into the feed. |
| 8. | Metabolism of the hormone in the animal body and mode of excretion | : | Like its natural hormone, it gets quickly metabolized and is excreted through urine. |
| 9. | Residues in animals and environment | : | The hormone, like the natural hormone, gets metabolized and more than 90 % of it is eliminated from the body within 24 hours. |
| 10. | Target organism | : | Fish and shell fish |
| 11. | Shelf-life | : | 90 days from the date of incorporation of the hormone into the feed. The hormone is photosensitive. |
| 12. | Uses | : | Used to produce all-female seed of fish |
| 13. | Recommended dosage | : | 20-120 ppm through diet. If it is through immersion 75-400µg/L. |

**9. Diethylstilbesterol**




| | | | |
|---|---|---|---|
| 1. | Hormone name, formula0 and structure | : | Diethylstilbesterol ($C_{18}H_{20}O_2$) M.W. 268 |
| 2. | Functional component | : | As such. |
| 3. | Mode of action | : | The hormone, when administered in small doses, stimulates growth. |
| 4. | Mode of application | : | Through feed. |
| 5. | Side effects in animals, if any | : | Not known. |
| 6. | Deleterious effects in the environment, if any | : | Not known. |
| 7. | Recommended preparations required for applying in aquaculture | : | Incorporation of hormone into the feed. |
| 8. | Metabolism of the hormone in the animal body and mode of excretion | : | It does not get metabolized quickly in the animal body. |

| | | |
|---|---|---|
| 9. Residues in animals and environment | : | The hormonal residues remain in the body for a long time. |
| 10. Target organism | : | Fish and shell fish |
| 11. Shelf-life | : | 90 days from the date of incorporation of the hormone into the feed. The hormone is photosensitive. |
| 12. Uses | : | Used to enhance growth in fish and shellfish |
| 13. Recommended dosage | : | 1-5 ppm through diet. However, its use in shrimp culture is not permitted. |

**10. Serotonin**

| | | |
|---|---|---|
| 1. Hormone name | : | Serotonin |
| 2. Functional component | : | The entire hormone. |
| 3. Mode of action | : | A neurotransmitter. |
| 4. Mode of application | : | Through feed |
| 5. Side effects, if any | : | A natural product with no known effect on ecology. |
| 6. Deleterious effects in the environment, if any | : | Not known |
| 7. Recommended preparations required for applying in aquaculture | : | Through feed |
| 8. Metabolism of the hormone in the animal body and mode of excretion | : | Readily metabolized and excreted through urine. |
| 9. Residues in animals and environment | : | Not known |
| 10. Target organism | : | Shell-fish |
| 11. Shelf life | : | A year at 2-6$^0$ C |
| 12. Uses | : | For improving gonadal development and inducing spawning in bivalves and crustaceans. |
| 13. Recommended dosage | : | Information not available. |

**Current list of hormones approved in the world**

| S. No. | Hormone | USA | Europe | Australia | Canada | Japan |
|---|---|---|---|---|---|---|
| 1. | HCG | Yes | - | Yes | - | - |
| 2. | MT | - | - | Yes | - | - |
| 3. | MDT | - | - | Yes | - | - |

In USA, the following hormones/hormone preparations have been registered as Investigational New Animal Drugs (INAD) and they are under various stages of evaluation for approval as New Animal Drug Application (NADA) by the FDA:

1) Common Carp Pituitary (CCP)
2) GnRH preparations (Ovaprim, Ovaplant and Repro Boost)
3) 17α-Methyltestosterone
4) 17β-Oestradiol

The FDA recognized that many food-producing animals in their early life stages are not normally used for human food. Therefore, it has been determined that specific drugs and drug claims may be considered to be of no risk to human food safety if that drug is proposed for use in the early life stages of an aquatic species, and:

1) There is no significant risk that harmful residues will be present in the fish when attaining marketable size as a result of treatment at the early life stage, and
2) The agency has no doubts about the use of the drug at later life stages.

In view of the above, FDA proposed to increase the availability of approved animal drugs for minor species and minor uses (MUMS document) and it has proposed draft legislation for approval by the Congress.

In Europe, the use of steroid hormones is not permitted since 1988. The European Commission published the views of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) in 1999. The SCVPH concluded that risks associated with the consumption of meat from hormone-treated cattle might be greater than previously thought. The World Trade Organization (WTO) Appellate Body ruled that the EU had not undertaken a proper risk assessment prior to imposing ban on import of meat from animals treated with hormone growth promoters. At the request of the Minister of Agriculture, Fisheries and Food, a Sub-Group to the Veterinary Products Committee (VPC) was asked to review the SCVPH report and the Committee's interpretation of the data is cited in the report. The group in its report submitted in October 1999, concluded that the likely levels of consumer exposure to 17β-oestradiol, progesterone, testosterone, zeranol and trenbolone resulting from their use as growth promoters were very low in comparison to the amounts of these hormones produced naturally by the bodies of some people. France is the only EU country that uses testosterone in farm animals.

In a number of Asian and South American countries, steroid hormones have been used to produce all-male tilapia seed for the past 25 years and all-male tilapia is exported to USA, Europe, Japan and many other countries. In general, in Europe and USA the admissible hormonal residue limit is less than one microgram per kilogram of meat. Hence, till date there has not been any rejection of all-male tilapia consignment by any of these countries.

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CHAPTER 8

# Vaccines and Vaccination in Fishes*

## Introduction

Since the last couple of years there has been a change in emphasis from disease treatment to prevention in aquaculture. This has been aided by the development of large-scale controlled environment production facilities and newer methods of immunization. The nature of environmental and nutritional diseases does not allow a wide choice of basic methods to combat them. Proper design of facilities and good management, including sanitation, are indicated for prevention of environmental diseases. To overcome nutritional diseases, fish requires correct amount of nutritionally balanced feed. However, with increasing intensification of fish culture and the changing environment, the number of infectious fish diseases are also on the increase. Therefore, research on the pathogenesis and pathology of these diseases, their prevention and control have become essential.

## Management of diseases

In India, freshwater aquaculture is largely based on farming of carps. Diseases of various kinds occur either as an outbreak or as sporadic incidences and hamper productivity and the cumulative effect of these are tremendous in terms of economic loss. Management of communicable diseases can be approached from several angles:

- Prevention of contact between pathogen and host
- Manipulation of environment to favour the host
- Prophylactic use of chemicals to remove pathogens
- Immunization to increase resistance to specific pathogens

## Immune response of fish to pathogens

Like other vertebrates, fish have both specific and non-specific responses against pathogens. Non-specific (innate) responses occur prior to the activation of specific immune responses; they include agglutinins, lysins, complements, lysozyme and C-reactive protein-like substances. These are effective in the mucous on the body surface and/or in the blood. Interferon in the blood inhibits viral replication; however, its role in parasitic infections is unknown. Non-specific phagocytic cells occur, including monocytes, granulocytes and thrombocytes; non-specific cytotoxic cells have also been found.

Fish produce immunoglobulins against particulate and soluble antigens. In all cases, intraperitoneal injection induces a higher antibody response than spraying or immersing the fish in antibody-containing water. Unlike the mammalian immune system, temperature is important in eliciting primary and anamnestic (immune memory) responses. Fish do not

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have lymph nodes– their antibody producing lymphocytes are in the spleen and anterior kidney and not in the thymus. The distinction between B- and T-lymphocytes in fish is thus not as clear as in mammals.

Specific phagocytosis by peritoneal cells and peripheral blood leucocytes is enhanced by antibody and complement as in mammals and piscine complement can be activated by both the alternate and classical pathways. It is similar to mammalian complement in that free calcium and magnesium ions are needed for lysis and lytic activity can be reduced by prior incubation in zymosan. Fish complement is also heat labile and some of its components show compatibility with mammalian components.

Most of what is known about the immunological response of fish to parasites is confined to studies of some of the more common pathogenic protozoa, many of which can cause mortality in fish. In some fish species, lack of susceptibility to certain parasites is due to non-specific complement mediated lysis. In many cases, however, susceptible fish that recover from infection show strong levels of specific acquired immunity. Catfish that recover from *lchthyophthirius* infection show protective immunity for up to eight months. This is due to immobilizing antibodies in the mucous, as well as in the blood, which is thought to immobilize the infective agent and prevent its entry.

Protective immunity against *lchthyophthirius* appears directed against the parasite's cilia. Catfish injected with cilia of a free-living flagellate *Tetrahymena pyriformis* were protected against *lchthyophthirius* and showed lower mortality on challenge than those injected with *lchthyophthirius cilia*. Inoculation of 5 mg of cilia protein conferred protection, but catfish inoculated with cilia-free antigen were not protected, nor was protection induced by oral drench or topical application.

Protective immunity against trypanosomes is by humoral antibody. For example, mortaility is often high in goldfish infected with *Trypanosoma danilewskyi* but depending on the size of the inoculum, 40-90 % of fish can recover and resist further challenge.

It appears that phagocytosis by peritoneal macrophages is most important during the chronic phase, but during the acute phase, parasitaemia can be controlled by complement fixing antibody. Fish that recover from infection resist challenge and passive transfer of lymphocytes from immune fish with antibody can confer partial protection. Complement-fixing antibody and cellular responses are also shown by summer and winter flounders infected with *Cryptobia bullocki*, and the humoral antibody may be responsible for the decline in prevalence of the parasite. Recovered fish are protected from challenge.

**Immune system**

The specific immune system comprises two basic components called humoral and cellular immunity. The humoral immunity is carried out by B lymphocytes whereas cellular immunity is by the T lymphocytes. B cells produce antibodies or immunoglobulins which are protein molecules that bind the foreign substances or antigens (harmful bacteria or viruses) in the bloodstream. These are then destroyed by various means like phagocytosis by macrophages.

T cells do not produce antibodies but they can recognize antigens bound to a type of molecule on the surface of a foreign cell with the help of a specialized molecule called receptor.

On primary exposure to an antigen, T and B cells cooperate in the response. A specific clone proliferates and matures into effector cells (B cells become plasma cells producing antibodies and T cells become cytotoxic or other effector cells or memory cells). This process constitutes primary response. On subsequent stimulation by the same type of antigen, the memory cells again multiply into effector cells or memory cells, the numbers of which then proliferate accounting for the stronger secondary response.

**Protection against pathogens through immunization**

In spite of recent research, there remain many gaps in our understanding of fish immune system. It is similar in many respects to that of mammals (*e.g.* production of specific immunoglobulins, phagocytic or cytotoxic cells) but has its own distinctive features (*e.g.* reduced number and structural differences in immunoglobulin classes, absence of lymph nodes).

In case of immunization, the objective is to elicit a good response to challenge producing a long lasting immunological memory, which is an effective and important factor than a strong primary response.

Immunity can be acquired by allowing infection to run its course where protection on recovery is long lasting and mortality to subsequent heavy challenge is reduced. Thus immunization by using small primary infections and/or less virulent pathogenic strains needs further study as a prophylactic measure. Moreover, the possibility of cross protection between non-pathogenic and pathogenic organisms with similar antigens may also be rewarding.

At present, although there are a few 'live' attenuated or killed vaccine available for some fish viral and bacterial diseases, no vaccine is available against protozoal diseases. Irradiated or killed protozoal vaccines do not confer protection, whereas successful use of crude antigen against lchthyophthiriasis shows that this is a viable possibility. The ability of a fish to develop immunity to a disease by vaccination appears to depend on three main factors:

- Size of the fish
- Water temperature
- Method of vaccination

Environment and hygiene can also affect immunity. The immune response to vaccination increases with fish size and with increasing water temperature.

There are three basic methods of vaccinating fish:

- Immersion
- Oral (in the feed)
- Injection

If the fish are exposed to a disease for 3 to 12 months, and if they weigh less than 50 grams, immersion vaccination may be the easiest and least expensive method. The fishes are dipped into a diluted, aerated vaccine solution for up to 60 seconds. Oral vaccines are used in the feed and in most cases are intended as a "booster" following immersion vaccination. Oral vaccination is appetite dependent and must be administered over a number of feedings. The cost is moderate, but the duration of immunity may be only two to four months. The effective protection is fair and a typical response is 20 to 50 % improvement in survival relative to unvaccinated fish.

**Development and administration of vaccine**

Many modern approaches to vaccine development against viral and bacterial organisms of medical and veterinary importance could be adopted for fish diseases. For example, protective antigens can be isolated, characterised, purified and synthesized biochemically or using DNA recombinant technology. The delivery system can be simplified by oral delivery in the feed– a practical approach to antigen delivery to fish of any size without imposing much stress on the host. Vaccines could also be delivered by hyperosmotic immersion, although this is more stressful or by spraying the antigen under pressure. Intraperitoneal injection of antigen is most effective but is a little cumbersome, time consuming, stressful and unsuitable for small fish. Vaccines are preparations of antigens derived from pathogenic organisms, rendered non-pathogenic by various means and aimed at stimulating the immune system in order to increase the resistance to disease from subsequent infection by pathogen. The two key elements of adaptive immune responses which vaccination seeks to exploit are: **specificity** and **memory**.

**Present status of vaccine development**

Significant strides have been made in our understanding of molecular mechanisms of disease, and in some cases this knowledge has resulted in the successful application of molecular genetics for vaccine design. However, in the aquaculture industry relatively little is known of either the host immunity disease and the pathogenesis of disease. Until these are elucidated, the successful application of biotechnology to fish vaccine industry will be largely a matter of chance. From the perspective of the vaccine manufacturing industry, there must be some guarantee of safety and efficacy prior to embarking on a genetic approach. Vaccines that are derived from genetically modified organisms are costly and time consuming to license and launch. Just as relatively little is known about fish immunity, its activation and effect or function, so too little is known about many of the fish diseases, particularly catfish. For example, enteric septicemia of catfish (ESC), caused by *Edwardsiella ictaluri* can decimate catfish ponds at almost any stage of production. Exactly where the bacterium comes from, how it becomes pathogenic and what triggers it to become pathogenic are not known. Also, very little is known about the nature of the protective antigens. Immunity against ESC is generally recognized as being cellular rather than humoral. Therefore, conventional methods of antigen detection relying on specific antibody (*e.g.,* ELISA or western blot), while detecting immunodominant antigens, may not elucidate the critical protective epitopes that result in generation of relevant, protective responses.

Clearly many vaccines have been produced for both human and animal health, wherein the nature of the protective mechanisms and antigens is not known. These products have relied on conventional methods for successful vaccination. They fall into two main categories, killed and modified live vaccines. Killed vaccines generally have been manufactured by growing the pathogen to high yields and treating them with an inactivating agent (*e.g.,* formalin, beta propriolactone, etc). Modified live vaccines may be generated by treating the pathogen with either a mutagenic agent, growing at different temperatures (to produce temperature sensitive mutants), or by attenuation through continuous growth in a different environment (*e.g.,* continuous cell culture).

Following the definition of a potential product, or the discovery of a new vaccine, feasibility studies may be carried out in order to prove the concept that the vaccine is protective. In its simplest form, proof of concept may comprise killing a pathogen and testing its efficacy. Alternatively, the pathogen may be attenuated so it is no longer harmful to the host species. Recombinant vaccines may comprise both killed (*e.g.,* expressed proteins), live or live vectored vaccines.

At present no vaccines for protection against bacterial, viral or parasitic diseases are commercially available in the country. Bacterins have been prepared by inactivating strains isolated from the specific pathogens to be treated. The choice of bacterial strains is important and there is some contention that vaccinated fish are not protected against challenge with heterologous isolates of *Aeromonas hydrophila*. For bacterin preparation, antigens responsible for inducing protective response need to be maintained during inactivation steps. Several studies demonstrate that the method of preparation of bacteria significantly influences the immune response. Some workers found that vaccines prepared from heated and disrupted cells induced a higher agglutinating antibody titer over formalin killed cells. The antiserum produced in response to formalin-treated and heated antigens cross reacted but were not identical. It is suggested that formalin treatment may alter the antigen structure and consequently processing of the antigens by the macrophages, while heating and breakage could release more antigenic material. The general conclusion is that attention must be paid to the preparation of the antigens for vaccinations, both with regard to the heterogeneity of the strains and the actual preparation of the bacteria. In many vaccination studies, the question addressed is whether or not fish respond immunologically using such indicators as antibody titers. Vaccination has rarely been assessed in terms of protection because of the difficulties in providing an effective challenge, particularly for field trials. Reports on challenge trials conducted in the laboratory are also complex to evaluate and compare because of significant differences in such things as the vaccine preparation used, the route of exposure, the method of challenge and the age, species and size group of fish used. Moreover, most trials of vaccination have not assessed the specificity of protection or its duration beyond a few weeks. Thus, where trials have indicated protection, it is not clear whether it is due to specific immunity or due to non-specific mechanism with memory component.

Vaccines are preparations of antigens derived from pathogenic organisms which are rendered non-pathogenic by various means in order to stimulate the immune system in such a way so

as to increase the resistance to disease from subsequent infection by a pathogen. A vaccine should be safe and potent inducing high level of protection.

Recent advances in biotechnology and immunology have revolutionized the development of vaccines for use in human beings and animals. Many of the techniques are highly sophisticated and in view of the cost of research and the relatively limited market for vaccines in fish farming, their applicability might be limited. AVL, the UK-based world leader in fish health has been granted a Marketing Authorisation in the UK and Northern Ireland for their product AquaVac™ ERM Oral for rainbow trout, an orally administered vaccine against Enteric Redmouth Disease. This is the first Marketing Authorisation to be awarded to an oral vaccine for fish in the UK, and AquaVac™ Vibrio Oral, which is licensed in Greece for seabass, as the only licensed oral vaccine for fish in Europe. ERM Oral is used as a booster vaccination to extend the protection conferred by immersion vaccination of fry with AquaVac™ ERM against Enteric Redmouth Disease, thus giving proven protection throughout the production cycle. AquaVac™ ERM Oral comes in the form of a liquid emulsion and is licensed for mixing into all standard production feed types and can be utilised on all fish farms.

The vaccine can be used in fish as young as 7 days and up to 31 days after hatching. Seven days is the youngest age at which catfish have been vaccinated to prevent infection. The vaccine can also be given by bath immersion on the transport vehicle that takes the young fry to the pond, or in tanks at the hatchery.

**Autogenous vaccines**

The United States Department of Agriculture has allowed the use of autogenous biologics (vaccines) within the aquaculture industry. Autogenous biologics are rapid response products for managing new disease outbreaks not yet controllable with licensed commercial biologics. These autogenous vaccines have been of considerable value for fish farmers by helping the growing industry manage new disease challenges.

Autogenous vaccines are a valuable aid to aquaculture and have received wide acceptance and use. In general, the following situation describes the use pattern for autogenous vaccine in fish culture:

- One or more production facilities are affected by a new disease problem causing sufficient losses of economic concern.
- Veterinarians, scientists and diagnostic laboratories identify the organism responsible for the losses.
- The isolated organism is cultured in quantity and prepared as an autogenous product by a licensed vaccine company.
- The autogenous biologic is used to protect remaining animals at risk on the particular affected farm or geographic location.

Autogenous biologics are interim solutions for managing disease problems until licensed products become available. These products need to be made available quickly following detection of disease problem for which no tested or proven licensed product is available.

Bacterins may be produced for delivering as immersion or injection product. Actual mode of delivery will be dependent on the disease in question. An autogenous vaccine may be delivered in conjunction with another licensed product, however, only under the approval of licensed veterinarian or registered aquaculture practitioner.

**DNA vaccines (Gene vaccine)**

DNA vaccines or gene vaccines are new tools being investigated for use in a growing number of health related fields, including aquaculture health. Traditional vaccines are made up of protein released from live or attenuated disease agents. These are injected into an uninfected host and trigger an immune defense response. DNA vaccine contains the gene of disease agents that codes for one or more of the proteins used in traditional vaccines. The DNA vaccine is injected into muscle tissue cells of a susceptible host, which stimulates them to produce the proteins (antigens) that trigger an immune system to recognize and attract an infection by the real pathogen. There are several advantages of the DNA vaccines over traditional vaccines, including lower risk of accidental infections using live or attenuated pathogens. DNA is also more stable than protein, making it hardier during the preparation process. Besides, DNA does not require 'cold chain', as necessary for protein based vaccine production. It is more stable for use under warm field conditions. DNA vaccines required to stimulate an immune response appear to be much lower than traditional vaccine. Three fish vaccines developed in collaboration with the Institute of Aquaculture, have been commercialised (Vibriosis, Enteric Red Mouth and Furunculosis). Recently, research has also commenced on the development of a DNA vaccine for bactrial kidney diseases.

**Vaccine against some important pathogens**

***1. Aeromonas hydrophila***

Due to its antigenic diversity, *A. hydrophila* presents problems in developing effective immunization programme for fish. There are two possible approaches for developing a vaccine for *A. hydrophila* with regard to origin of the antigen. One is to employ and isolate from a particular farm and make a monovalent vaccine for that particular strain of organism. Such a vaccine will have a limited application. Some workers have tested the efficacy of this type of bacterin by intraperitoneal injection or hyperosmotic infiltration immersion immunization. Although the results were inconsistent, some vaccinated fish demonstrated significant protection and increased weight gain.

A second method of preparing a vaccine is a polyvalent form prepared from many different isolates of *A. hydrophila*. A polyvalent vaccine may have to contain all the antigens (isolates) that the fish may encounter.

The heterogenicity of *A. hydrophila* is not the only problem confronting successful vaccination of fish with this organism. Method of vaccine preparation and its application,

age and size of the fish also present problems. Several vaccine preparations such as whole cell antigen, attenuated or heat or formalin killed, sonicated extracellular products and bacterial soluble extracts have been used. The sonicated preparation resulted in significantly higher agglutination titres than either the whole cell or freeze-thaw preparations.

### 2. *Edwardsiella tarda*

*E. tarda* is a frequent pathogen in cultured species of fish. This is an enteric pathogen and is commonly found in the intestine. The protective immune mechanism against *E. tarda* appears to be a combination of humoral and cell medicated immunity. Researchers have demonstrated a high humoral antibody titre in fish after vaccination with heat or formalin killed *E. tarda*.

Immune response of whole cell and cell sonicate intraperitoneal injection and whole cell hyperosmotic infiltration and immersion has been tested. Intraperitoneal injection produces highest antibody titre in 2-6 weeks and shows greatest protection upon challenge. Dipping in the vaccine without hyperosmotic shock results in reduced antibody titre. Based on several experimental studies, the following conclusions have been drawn in the case of *E. tarda* vaccine.

i. The best immune response is obtained in a bacterin with $10^8$ cells/ml,

ii. An immersion time of 3 minutes is required to induce immunity,

iii. Although a single exposure to the bacterin is effective, a better response is observed with a triple exposure, and

iv. Effective immunization can be induced at a temperature of 29-33$^{o}$C.

### 3. *Vibriosis*

Vibriosis is a widely distributed disease and affects many fish species. Pathogenesis of vibrios concerns the mode and site of entry of the organisms in fish. Although very few studies have been undertaken in this regard, the most possible route of entry is supposed to be the gill. Other possible sites of entry are of course the skin and gastrointestinal tract. Vibriosis is usually characterized by haemorrhagic septicaemia, anaemia and leucopenia.

Four methods of vaccination are available *i.e.* by injection, by feeding (in the diet), by immersion and by spraying the vaccine on the fish.

Injection using repeater syringes is used to immunize large number of fish through intraperitoneal route. Disadvantages of the injection technique are that it imposes stress on fish due to handling. It cannot be used conveniently with small fish and mass vaccination operation requires a large workforce. The advantages of the technique are that it provides the highest level of immunity; it utilizes the antigen efficiently and provides an opportunity for using adjuvants to enhance and prolong immunity.

Oral vaccination has received considerable attention because it can be carried out whenever required and it does not involve stressful handling. The technique, however, results in uneven immunization because individual fish may consume different amounts of the vaccine. It is also wasteful because much of the vaccine is destroyed by the gastro-intestinal tract. The immersion and spray vaccinations are methods of choice for mass immunization operations and the delivery of the vaccine is by applying it to the external surface. The efficacy of the direct immersion (DI) method is amply proved in laboratory and field tests.

***4. Ichthyophthiriasis***

Immunology of fish to protozoan or helminth parasites has received very little attention compared to viral and bacterial pathogens. Although ichthyophthiriasis is not the most frequently reported parasite of fish, it is one of the most important and devastating one. 'Ich' is a problem not only because of its pathogenic capability, but also for its life cycle, which includes a host stage and a free living multiplication stage. The virulence of *I. multifilis* is beyond doubt, however, the pathogenicity is largely dependent on water temperature, which is greatest between $20^{o}$-$26^{o}$ C.

Experiments have been conducted by injecting homogenized trophozoites of *I. multifilis* in fish to find out agglutinating antibody titres. Fish injected with homogenized trophozoite preparation without adjuvant have shown titres of 1:2560 compared to the trophozoite with adjuvant of 1:5120. Some of these studies have indicated that antigens of this parasite confer protective immunity when properly administered. Immunization stimulates the production of agglutinating antibodies, which are located in the external mucus, although the mechanism of protection is not known very clearly. However, as the tomites come in contact with the mucus they get immobilized, preventing infection.

Attempts of vaccination by intramuscular injection, oral drench and topical application have proved that the former method is most effective. Fishes were protected against *I. multifilis* after 4 weeks, but maximum protection was achieved 10 weeks after vaccination by whole cell or ciliary bath.

**Licensing procedure**

The FDA regulates pharmaceuticals and feed additives, and the USDA-APHIS has defined the procedure for licensing biological products in the USA. These regulations are documented in the Code of Federal Regulations. However, in consideration of biotechnology-derived products, the USDA-APHIS requires a number of studies that assess environmental impact. This 'Risk Assessment' documentation is pivotal to the successful licensure of any biological agent (live or killed) that is derived from a genetically modified organism (GMO). However, these procedural details and regulations need to be implemented in India as well as in other Asian countries.

## Conclusion

Despite the lack of knowledge, fish vaccination appears as an essential method of controlling infectious diseases in intensive fish culture. Since there is no absolute control method for a defined disease, immunoprophylaxis must be combined with other preventive control activities such as hygiene, genetic improvement, health monitoring and certain forms of chemical treatment. The extension of research into the pathogenesis of infectious diseases and the immunity of fish to these infections with particular emphasis on research on the local immunity, cell mediated immunity, molecular basis of virulence and immunogenicity of the pathogens can improve vaccination programmes, which have to be seriously undertaken in the country.

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CHAPTER 9

# Probiotics in Aquaculture*

The term probiotic is attributed to organisms and substances, which contribute to intestinal microbial balance. Although a strict definition of probiotics is difficult to come by, they are defined as "*living microbial cells administered as dietary supplements with the aim of improving health*". Gatesoupe (1994) reviewed the state of probiotic usage in aquaculture and stated that the first application of probiotics in aquaculture is relatively recent, but the interest in such environment-friendly treatments is increasing rapidly. Only a few scientific papers deal specifically with use of probiotics in aquatic animals. More questions have been raised as to whether probiotics have any relevance to the aquatic environment. Aquatic animals are quite different from land animals for which the probiotic concept was developed.

Recognizing the conceptual difference of terrestrial and aquatic probiotics, Gatesoupe (1994) suggested a modification in the definition of probiotics, as used in aquaculture. He defined probiotics as *microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health*. He further classified the microbial preparations used in aquaculture into three types– biocontrol agents, probiotics and bioremediation agents. Biocontrol agents are those methods of treatment using the antagonism among microbes to kill or reduce the number of pathogens in the aquaculture environment. Those bacterial treatments which improve the water quality and thus indirectly the production were termed as bioremediation agents. Recently, several commercial products have sought to exploit the idea that bacteria, which improve water quality, may be beneficial to animal health. Among shrimp farmers in India, these products are known as *water or soil-probiotics* and most of them contain nitrifying bacteria and/or *Bacillus* spp. The commercial availability of probiotics and bioremediation agents in shrimp culture and its widespread usage in India has spawned separate terminologies among shrimp farmers. The strict probiotic agents are known as gut-probiotics and the bio-remediation agents are known as water-probiotics.

Probiotics have been classified as biological preparations under the proposed Aquaculture Drug Regulations of India. An 11-point criterion has been set out to regulate the use of these preparations. The following account details some of the well studied probionts tried in aquaculture. A cursory examination shows that there is little information on the mode of action of the strain used. Other criteria, for which scanty information is available are, 1) impact on the environment and side effects in the animal, and 2) fate of the organism in the animal body/aquatic environment.

The use of probiotics in aquaculture is a clear case where commercial use started before there was research backing to support its use. Time and again, companies have borrowed

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the probiotic preparations used in animal (veterinary) husbandry, and are directly applied in to aquaculture systems. So far, adverse impacts on animal health and environment have not been reported, but reports of lack of any concrete benefits are many.

Based on the above, it is recommended that 1) more funding support be given to research on use of probiotics in aquaculture, 2) more research be directed to investigate the fate of probiotic organisms in the environment and in the animal and impact if any on the environment and animal, 3) commercial probiotic manufacturers and marketers should ensure that they comply with and disclose all the criteria set by the Aquaculture Authority, and 4) commercial probiotic manufacturers and marketers should get their products tested by a competent research laboratory before they market their product.

**1. *Lactobacillus***

**Origin/Status/Description:**

Gram positive rods varying from long and slender rods to short coco-bacilli, usually non-motile and non-sporing. Fermentative metabolism, at least half the end product from carbohydrate metabolism, is lactate. Saccharoclastic, nitrate reduction unusual, catalase negative, lack cytochromes, no gelatin liquefaction and casein not digested. Indole and $H_2S$ production absent. Pathogenicity unusual. G+C content of DNA range from 34.7 ± 1.4 - 53.4 ± 0.5 moles %. Distribution is universal. The species has been reported from fresh and brackish water organisms, but not from marine organisms.

***Mode of action***

Many species of this genus are known to produce inhibitory substances that have broad-spectrum antibacterial activity. Lactobacilli can produce compounds that inhibit the growth of microorganisms. *Lactobacillus* produce large amounts of $H_2O_2$ and also reduces the pH of the medium with the production of organic acids such as lactic acid, acetic acid and Diacetyl. Once inside the gut of the host, Lactobacilli attach themselves to the epithelial cells in the gut wall. Competition for adhesion receptors with pathogens might be the first probiotic effect. Colonization of the digestive tract by bacteria capable of producing lactic acid through fermentation inhibits the proliferation of putrefactive microbes in the gut. *Lactobacillus* produces bacteriocins (bactericidal or bacteriostatic peptides), which have narrow range of antibacterial activity, mostly active against Gram positive bacteria. Lactocin produced by *L. acidophilus* is active against Gram negative and Gram positive bacteria. Acidolin, Acidophilin and Lactocin-F are the other antibacterial compounds produced by *L. acidophilus,* which are antagonistic to *Lactobacillus* species only. *L. plantarum* produces three types of bacteriocins, *viz*., Plantaricin A, Lactolin and Sakasin-A. *L. helveticus* is reported to produce Helvectin-J and Lactolin-27, which are antagonistic against other *Lactobacillus* species. Lactobacilli are also reported to play a role in stimulating the non-specific immune system of host.

***Target organism***

The antibacterial compounds produced by *Lactobacillus* have a broad spectrum of activity against bacteria that are closely related to the producer. It has been shown that *L. plantarum*

produces inhibitors against *Vibrio* sp. when the culture was grown in the presence of *Bacillus thuringiensis*. But there have been reports showing the effectiveness of these strains against Gram negative strains. *L. bulgaricus* is able to neutralize the toxin produced by *E. coli* under *in vitro* conditions. Ruterin produced by *L. plantarum* is antagonistic to Gram positive and Gram negative bacteria, yeast and *Trypanosoma cruzi*. The *Lactobacillus*/*Carnobacterium* strain isolated from rotifers increased the resistance of turbot larvae against a pathogenic *Vibrio* sp. *L. rhamnosus* was found to be effective against furunculosis in rainbow trout. The addition of *Lactobacillus* decreased the count of *Pseudomonas* sp. in oysters.

***Impact on environment/animal***

The beneficial effects of Lactobacilli have been described. The pathogenicity of the species has also been reported. The genus *Lactobacillus* has been reported to produce kidney disease in Pacific salmon. Since the species is nutritionally fastidious, it is not expected to survive in the natural environment, even under anaerobic conditions.

***Fate of the organism in animal/environment***

Some strains can colonize the gut and it is possible to maintain high population level in the gut through regular intake with food.

***Mode of application/recommended dosage***

i). Direct application to rearing medium in the case of filter feeding larvae and live feeds.

ii). Through enriched live feeds in the case of larger and prey-capturing larvae.

iii). By coating preparations on to pellet feeds in the case of juveniles and adults.

iii). Recommended dosage varies with species and stages in the life history of host.

***Implications on human health***

No negative impacts reported.

***Viability/storage***

Storage below 30° C recommended. Viability depends on the species.

**2. *Lactococcus***

**Origin/status/description**

Ubiquitous. Gram positive cocci, usually non-motile, non-sporulating that produce lactic acid as a major or sole product of fermentative metabolism and are generally catalase negative and lack cytochromes.

***Mode of action***

Lactococci produce three types of bacteriocins– Nisin, Diplococcin and Lactostrepcin. Nisin is produced by *L. lactis* and exists in five forms, *viz.,* Nisin A, B, C, D and E. Nisin A is the most active among these compounds. Nisin has a narrow spectrum of inhibitory activity affecting only Gram positive bacteria, including lactic acid bacteria like

*Streptococcas, Bacillus* and *Clostridium*. Possible cause for resistance of microorganism to Nisin is the production of the enzyme nisinase. Nisin acts as a surface active detergent. The sensitive microbes are adsorbed to the nisin producer followed by cytoplasmic membrane rupture and release of cytoplasmic material. The other compounds, Lactostrepcin and Diplococcin are antagonistic only to Gram positive bacteria.

***Target organism***

The species is mostly active only against Gram positive organisms. The bacteriocins produced by *L. lactis* showed some resistance to infections caused by *Listeria monocytogenes*, *Aeromonas hydrophila* and *Staphylococcus aureus*. *L. lactis* enhanced the growth of the rotifer *Brachionus plicatilis* and had an inhibitory effect on *Vibrio anguillarum*.

***Impact on environment/animal***

The species has shown to be effective in enhancing growth rate and immunity in the host. The pathogenicity of the species has also been reported. The species *Lactococcus garvieae* has been reported to produce disease conditions in yellowtail and rainbow trout. *L. piscium* has also similar effect on rainbow trout. Since the species is nutritionally fastidious, it is not expected to survive in the natural environment, even in anaerobic conditions.

***Fate of the organism in animal/environment***

Some strains can colonize the gut, but it is possible to maintain high population level in the gut through regular intake with food.

***Mode of application/recommended dosage***

1. Direct application to rearing medium in the case of filter feeding larvae and live feeds.
2. Through enriched live feeds in the case of larger and prey-capturing larvae.
3. By coating preparations onto pellet feeds in the case of juveniles and adults.
4. Recommended dosage varies with species and stages in the life history of host.

***Implications on human health***

No negative impacts reported.

***Viability/storage***

Storage below 30° C recommended. Viability depends on the strain.

**3. *Pediococcus***

**Origin/status/description**

Universal. Gram positive cocci occurring in pairs or in tetrads. Single cells or chains are rare. Non-motile do not form endospores. Fermentative metabolism, homolactic, producing D-l lactic acid. Catalase negative, nitrate not reduced, gelatin not liquefied. Acid but no gas from glucose, fructose, mannose, sorbitol and starch not fermented. Microaerophilic, G+C content of DNA ranges from 3444 moles %.

***Mode of action***

*Pediococcus* is known to produce bacteriocins which are inhibitory to broad spectrum of Gram positive microorganisms. The bacteriocins are Pediocin-A, Pediocin-PA1 and Pediocin-ACH. Other inhibitory substances produced by the genus are lactic acid and $H_2O_2$.

***Target organism***

The bacteriocins produced by *Pediococcus* are active against a broad spectrum of Gram positive species. The species inhibit the growth of *L. monocytogenes*. The pediocins are antagonistic to *Lactobacillus, Leuconostoc, Micrococcus, Streptococcus* and do not have any effect on Gram negative bacteria.

***Impact on environment/animal***

An increase in growth rate in Pollock larvae when fed with enriched *Artemia* metanauplii containing a preparation with *Pediococcus acidilactici* has been reported. Impact on environment not reported.

***Fate of the organism in animal/environment***

Some strains can colonize the gut, but it is possible to maintain high population level in the gut only through regular intake along with food. Fate in environment not yet studied.

***Mode of application/recommended dosage***

i). Direct application to rearing medium in the case of filter feeding larvae and live feeds.

ii). Through enriched live feeds in the case of larger and prey-capturing larvae. *Artemia* nauplii were enriched with self-emulsifying concentrate of essential fatty acids DHA Selco (INVE™, 150 mg.$l^{-1}$) with ca. 10 CFU $l^{-1}$ of *P. acidilacti*.

iii). By coating preparations on to pellet feeds in the case of juveniles and adults.

iv). Recommended dosage varies with species and stages in the life history of host.

***Implications on human health***

No negative impacts reported.

***Viability/storage***

Storage below 30° C recommended. Viability depends on the species.

**4. *Leuconostoc***

***Origin/status/description***

Universal. Gram positive cocci, spherical but often lenticular, usually in pairs and chains. Non-motile, fermentation of glucose with the production of D (-) lactic acid, ethanol and $CO_2$. Catalase negative, non-proteolytic, indole not formed. Arginine not hydrolysed, nitrate not reduced, non-haemolytic, G+C content of DNA ranges from 43-44 moles % for *L. lactis* and 38-42 % for all other species.

***Mode of action***

Inhibition of pathogens by *Leuconostoc* has been attributed to the production of organic acids such as lactic and acetic acid and diacetyls. *Leuconostoc* also produces some unknown bacteriocins, which are antagonistic against *Listeria monocytogenes*. The presence of *Leuconostoc* spp. associated with epithelial mucosa of stomach, small and large intestine of Arctic charr when fed with different dietary polyunsaturated fatty acid has been demonstrated.

***Target organism***

*Listeria monocytogenes*

***Impact on environment/animal***

Not reported

***Fate of the organism in animal/environment***

Not Reported

***Mode of application/recommended dosage***

i). Direct application to rearing medium in the case of filter feeding larvae and live feeds.

ii). Through enriched live feeds in the case of larger and prey-capturing larvae.

iii). By coating preparations on to pellet feeds in the case of juveniles and adults.

iv). Recommended dosage varies with species and stages in the life history of host.

***Implications on human health***

No negative impacts reported.

***Viability/storage***

Not Reported

**5. *Carnobacterium***

**Origin/status/description**

Straight to slightly curved Gram positive rods. Non-motile, non-acid fast, mixed respiratory and fermentative carbohydrate metabolism. Aerobic and facultative anaerobic, catalase positive. Reported G+C content of DNA varies from 52-68 %. Widely distributed in nature.

***Mode of action***

*Carnobacterium* strains produce bacteriocins (Carnocin) or bacteriocin like substances active against other Carnobacteria and lactic acid bacteria. *Carnobacterium piscicola* strain *CP5* was found to produce a bacteriocin named carnocin CP5 that inhibited *Carnobacterium, Enterococcus and Listeria* spp. In another study *C. piscicola* strain U149 was found to produce a bacteriocin, carnocin U149. Carnocin U149 was active against a large number of lactic acid bacteria including *Carnobacteria, Lactobacilli, Pediococcus,* and *Lactococcus.*

***Target Organism***

*Carnobacterium piscicola, Aeromonas hydrophila, A. salmonicida, V. anguillarum, V. salmonicida, V.ordalli, Proteus vulgaris, L. monocytogenes, Flavobacterium psycrophillum, Photobacterium damselae, Streptococcus millieri.*

***Impact on environment/animal***

The species is found to have high colonization properties. *Carnobacterium* species designated as strain K1 was found to adhere non-specifically to rainbow trout intestinal mucous and multiply readily in intestinal mucous and faeces. Colonization of large intestine in Arctic charr by *C. divergence* and true colonization of the intestine of Atlantic cod fry by *C. divergence* displacing other potential colonizers have been reported. Impact on environment has not been studied.

***Fate of the organism in animal/environment***

*Carnobacterium* spp. is suspected to multiply in the gut of rainbow trout juveniles fed with the strain and can persist there for three days.

***Mode of application/recommended dosage***

i). Direct application to rearing medium in the case of filter feeding larvae and live feeds.

ii). Through enriched live feeds in the case of larger and prey capturing larvae.

iii). By coating preparations on to pellet feeds in the case of juveniles and adults.

iv). Recommended dosage varies with species and stages in the life history of host.

***Implications on human health***

No negative impacts reported.

***Viability/storage***

Not reported

**6. *Bacillus***

***Origin/status/description***

The members of the genus *Bacillus* are all strict or facultative Gram positive aerobes. Many form chains or filaments and are motile with lateral flagella. They are spore formers (heat resistant endospores) and classified on the basis of the shape of spores. Fermentation process involves the production of large number of compounds among which organic acids predominate. The G+C content of the DNA ranges from 32-62 moles %.

***Mode of action***

*Bacillus* species are known to produce different kinds of antibiotics, such as Bacitracin, Polymixin, Trycodin, Gramicidin and Circulin, especially in relation to sporulation process. The genus has the ability to secrete many enzymes that degrade slime and bio films and allow antibiotics to penetrate slime layers around Gram-negative bacteria.

Further, *Bacillus* competes for nutrients and thus inhibits other bacteria from growing rapidly so that any resistant bacteria may not be able to multiply readily and transfer mutant genes. *Bacillus* NM-12 produces an antibacterial substance, a heat liable siderophore having a wide antibacterial spectrum against intestinal bacteria of coastal fish and that substance might be useful to control bacterial population in both fish intestine and culture water.

***Target organism***

Many authors have reported the antagonistic effect of *Bacillus* species against Gram-negative microorganisms. A decrease in Vibrionaceae group when rotifers were fed with spores of *Bacillus* spp. is reported. A decrease in *Vibrio* and other species both in shrimp gut and culture water were noticed when *P. monodon* larvae were given *Bacillus* S11. *B. toyoi* was found to be antagonistic to *Edwardsiella tarda* when fed to *A. anguilla* at the initial challenge stage. Antibacterial activity of *Bacillus* NM12 against *Acinetobactor* spp., *Bacillus* spp., Corneyform group Enterobacteriaceae, *Flavobacterium* spp., *Moraxella* spp., *Pseudomonas* spp., and Vibrionaceae has been demonstrated. Selected strains of *Bacillus* species were used in shrimp aquaculture ponds and these were capable of changing bacterial species composition in ponds and were effective in controlling *V. harveyi* population.

***Impact on environment/animal***

The *Bacillus* species was found to enhance the immune system and growth rate in the host animal. *B. toyoi* isolated from soil reduced the mortality in Japanese eel that were infected by *Edwardsiella* species and increased growth rate in Yellow tail. Administration of *B. toyoi* in turbot larvae led to an increase in growth rate. Increase in both growth rate and survival of *P. monodon* when fed with *Bacillus* S11 isolated from shrimp ponds were noticed. *Bacillus* species also increased survival and production of channel catfish.

***Fate of the organism in animal/environment***

Natural flora of aquatic organisms consists several species of *Bacillus*. *Bacillus* S11 was able to colonize and proliferate in the gut of *P. monodon* larvae. No specific study on the fate of the *Bacillus* species introduced into the environment has been made.

***Mode of application/recommended dosage***

Either as cultured cells of $3.0 \times 10^{13}$CFU/g or $3 \times 10^{10}$ CFU/ml and lyophilized cells of $5 \times 10^{12}$/g after mixing with pelleted feed.

***Implications on human health***

No adverse effects were reported.

***Viability/storage***

Slant culture and lyophilized cells remain viable at $4^0$ C.

**7. *Vibrio***

***Origin/status/description***

Straight or curved Gram negative rods, 0.5-0.8 μm in width and 1.4-2.6 μm in length. Facultative anaerobes capable of both fermentative and respiratory metabolism. Motile with polar or lateral flagella. Sodium ions are an absolute requirement for most of the strains. Fermentation of D-glucose results in acid but no gas. Oxidase positive. The G+C content of DNA ranges from 38-51moles %. Distribution is universal, found in aquatic habitats having wide range of salinities.

***Mode of action***

Some strains of *Vibrio* are known to produce siderophores that have a probiotic effect on the host. Non-pathogenic bacteria which produce siderophores are promising candidate probiotics against pathogens with low iron uptake capacity. It is reported that the inhibitory effect of *V. anguillarum* against *V. ordalii* is due to a siderophore. The probiotic effect of a *Vibrio* species designated as *Vibrio E* has been reproduced by the purified bacterial siderophore defroxamine. Competitive exclusion of other pathogenic bacteria is also suggested as another mode of action.

***Target organism***

Some species were found to be antagonistic against Gram-negative strains. A strain of *V. alginolyticus* strain is reported to be antagonistic against *V. ordalii, A. salmonicida* and *V. anguillarum*. A decrease in the population of *V. parahaemolyticus* in *P. vannamei* culture tanks treated with *V. alginolyticus* has been observed.

***Impact on environment/animal***

The probiotic strains were found to be beneficial in enhancing the immune system and growth rate in the host animal. A strain, *Vibrio E* was found to improve the resistance of larval turbot against *V. splendidus* and *Vibrio parahaemolyticus*. However, it has to be remembered that *Vibrio* species are well known pathogens causing diseases in larval and adult stages of shrimps and other cultivable organisms

***Fate of the organism in animal/environment***

In the marine habitat the species are known to dominate the microbial flora. Fate of the introduced *Vibrios* was not studied.

***Mode of application/recommended dosage***

Direct application of bacterial cultures having concentration of 0.5 MacFarland Standard ($10^6$ CFU/ml) to the rearing tank has been reported.

***Implications on human health***

*V. cholera* and *V. parahaemolyticus* is a well known human pathogen and, therefore, the species and strain to be used has to be carefully selected.

***Viability/storage***

Fresh cultures are used. Storage pattern not reported.

**8. *Aeromonas***

***Origin/status/description***

Gram negative rods. Motile by polar flagella, generally monotrichous. Facultative anaerobes, metabolism both fermentative and respiratory. Oxidase and catalase positive.

***Mode of action***

*A. media* has been reported to have the ability to produce BLIS (bacteriocins-like inhibitory substance), which is antagonistic *in-vitro* to a number of Aeromonads including *A. hydrophila* and a number of *Vibrio* spp. *In-vivo* inhibitory effect of *A. media* has also been demonstrated in oyster larvae against *V. tubiashii*.

***Target organism***

*Aeromonas caviae, A. hydrophila, A. salmonicida, Vibrio alginolyticus, V. cholera, V. harveyi, V. parahaemolyticus, V. pelagicus, V. tubiashii, Yeresina ruckeri*.

***Impact on environment/animal***

Prevented mortality to oyster larvae caused by an artificial infection by *V. tubiashii*. No impact on environment reported.

***Fate of the organism in animal/environment***

*A. media* could not be detected in the larval culture 96 h post administration. Fate of organism in environment has not been studied.

***Mode of application/recommended dosage***

In larval rearing tanks a concentration of $10^4$ cfu/ml has been reported as the effective dosage.

***Implications on human health***

Non-pathogenic to humans as the strain is psychrophilic.

***Viability/storage***

Not reported.

**9. *Saccharomyces boulardii***

***Origin/status/description***

Non-pathogenic yeast, isolated from Lychee fruit in Indo-China. The species is able to utilize glucose, glycine, maltose, saccharose, trehalose and raffinose.

***Mode of action***

The species has been reported to show a putative immunotherapeutic effect against a wide variety of diarrhoeal infections. Increased secretary immunoglobulin A and secretary components of other immunoglobulins in the small intestine of rat treated with *S. boulardii* has been reported. The organism is found to remove toxin receptors by way of protease activity and also produces a protease that reduces or removes brush border

glycoproteins involved in adhesion of pathogens to mucosa. Studies show that *S. boulardii* secretes a protein, which decreases the concentration of cholera toxin induced cAMP in epithelial cell lines.

***Target organism***

The effectiveness of the species in the treatment of diarrhoeal diseases (caused by *Clostridium difficile* and *Vibrio cholera*) in humans and farm animals is well documented and commercial formulations are available for the treatment of antibiotic associated diarrhoea. *S. boulardii* fed artemia nauplii and *P. monodon* zoea and mysis larvae were found to be resistant to an artificial *V. harvey*i infection.

***Impact on environment/animal***

An increase in disaccharide activity in humans and rats when *S. boulardii* was given through oral administration has been reported. Impact on environment not yet studied.

***Fate of the organism in animal/environment***

The species is able to colonize the digestive tract of farm animals and humans. *S. boulardii* is able to achieve high concentrations in the colon quickly, maintain constant levels, does not permanently colonize the colon and does not translocate easily out of the intestinal tract.

***Mode of application/recommended dosage***

i). Direct application to rearing medium in the case of filter feeding larvae and live feeds are recomended. *Artemia* nauplii enrichment at $10^4$ cfu/ml and *P. monodon* zoea and mysis fed at $10^5$ cfu/ml have been found to be ideal.

ii). Through enriched live feeds (at above concentrations) in the case of larger and prey- capturing larvae.

iii). Recommended dosage varies with species and stages in the life history of host.

***Implications on human health***

The species was found to show a putative immunotherapeutic effect against infections in humans.

***Viability/storage***

Storage in lyophilized form at 4° C has been recommended.

**10. *Alteromonas***

***Origin/status/description***

Common inhabitants of coastal waters and open oceans. The genus is composed of Gram negative curved or straight rods. Cells motile with polar flagella. Oxidase positive, capable of respiratory but not fermentative metabolism and do not form mycrocysts or endospores. Lack arginine dihydrolase system and all require seawater base for growth.

***Mode of action***

*Alteromonas* sp. (strain 1-2) was found to liberate into culture medium an antifungal compound 2, 3-indolinedione (also known as isatin*)* in large quantities inhibiting the growth of pathogenic fungi. In another study *Alteromonas* like bacterium (strain No. 6) isolated from black tiger shrimp was found to produce a thermolabile protein with high molecular weight exhibiting antibacterial activity.

***Target organism***

This species was able to deter infections by the pathogenic fungus *Leginidium calinectes* in *Palaemon macrodactylus* embryos. An *Alteromonas* strain No. 6 showed inhibitory activity against 50 strains of vibrios including V. *parahaemolyticus, V. fluvialis, V. alginolyticus,* and *V. harveyii*.

***Impact on environment/animal***

Embryos of prawn reinoculated with the *Alteromonas* sp. or treated only with 2, 3-indolinedione survive better compared to bacteria free embryos, when exposed to pathogens. No deleterious effect on the environment has been reported so far.

***Fate of the Organism in animal/environment***

The species was found to colonize the surface of *P. macrodactylus* embryos and protect the embryos from pathogen invasions. Fate of the organism was not studied.

***Mode of application/recommended dosage***

The embryos are treated with pure cultures (concentration not specified).

***Implications on human health***

None reported so far.

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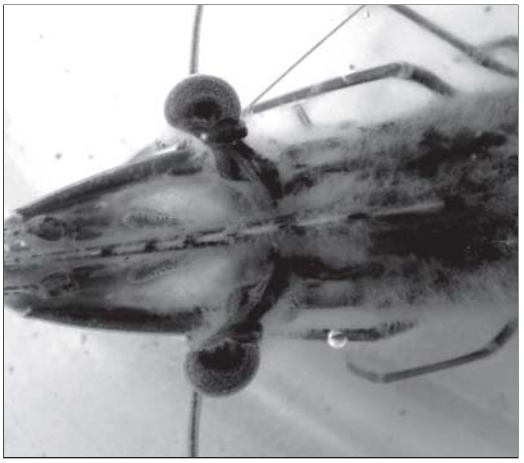

CHAPTER 10

# Immunostimulants in Aquaculture*

## 1.1. Introduction

Aquaculture is a fast growing industry and the frequent outbreak of diseases, especially of viral origin, is a major setback to the sector causing huge economic loss. Microorganisms that cause massive disease outbreaks in fish and shrimp culture systems are mainly opportunistic pathogens, which invade when the host is weakened or stressed by adverse environmental conditions. The use of antibiotics and other chemotherapeutants leads to the development of resistant pathogenic strains besides causing severe ecological damage to the culture environment. In this context, the use of prophylactic agents such as immunostimulants deserves much attention. Immunostimulants may increase the resistance level sufficiently to prevent infection by opportunistic pathogens and, therefore, lead to improved performance, enhanced growth and reduced mortality throughout the production period.

Immunostimulants are chemical compounds that activate the immune system of animals and render them more resistant to infections by viruses, bacteria, fungi, and parasites. Cell wall fragments of microorganisms render animals more resistant to microbial infections. The ability of the immune system to respond to microbial surface components is the result of an evolutionary process whereby animals have developed mechanisms to detect common and highly conservative chemical structures of potential pathogenic microorganisms and to use those structures as ''alarm signals'' to switch on defense against infection. The immune system will, therefore, respond to an immunostimulant as if challenged by a pathogenic microbe. Administration of an immunostimulant prior to an infection may thus protect the animal against an otherwise severe or lethal infection.

Shrimps and other invertebrates have immune systems that are less well developed than in fishes. Although shrimps may become immune after 'vaccination' by exposure to killed preparations of a pathogen, they are primarily dependent on non-specific immune processes for their resistance to infection. Increasing resistance to a specific pathogen may not work and, therefore, increasing the non-specific immunity of the shrimp to equip them with broad-spectrum defensive ability is the appropriate strategy. It should, therefore, be expected that immunostimulants may become an important tool to reduce diseases of crustaceans in culture systems. Immunostimulants have been shown to induce both immune related and non-immune related effects in shrimps. Immune related effects include enhanced phagocytic activity, activation of proPhenol oxidase activity and increased clotting of haemolymph. Non-immune related effects include higher growth and survival, enhanced tolerance to water temperature, and higher tolerance to salinity and stress.

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The immunostimulants present in the cell walls of mushrooms and yeasts are mainly β 1-3 glucans. The active principles of immunostimulatory bacterial cell wall preparations are various muramyl peptide fragments, lipopolysaccharides (LPS) and acyloligopeptides.

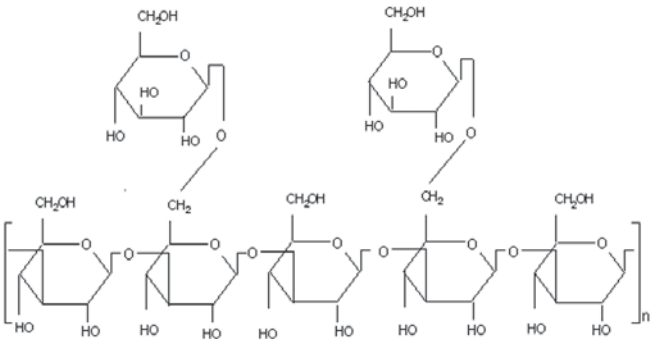
## 1. 2 Microbial derivatives

### 1.2.1 Products from yeasts/fungi

**Glucans**

β-1,3 glucans appear to be the most promising of all immunostimulants so far examined in fish and shrimp. β glucans are poly glucose molecules linked through β- 1,3 bonds in a long chain and with β-1,6 branches consisting of single glucose molecule or chains of glucose molecules. Such glucans can exist in various structural forms and may be in the form of water-soluble oligomers, water soluble or insoluble macromolecules or particulates.

***Structure of β - 1,3 Glucan***



There are well defined receptors for β-1,3 glucans on the macrophages of warm blooded animals, fish and on shrimp haemocytes. The β-1,3 glucan receptor on macrophages is highly specific for the β-1,3 glucan structure as the uptake of β-glucan particles in human or fish macrophages is inhibited only by oligopolysaccharides with the β-1,3 glucan backbone, not by other carbohydrates. The β-1,3 glucan receptor on macrophages "recognizes" a β - 1,3 glucan chain with more than 3 to 5 glucose units.

Glucan is from an evolutionary point of view, the most widely and most commonly observed macrophage activator in nature. β-1,3 glucan has been proven to both stimulate and activate macrophages, which can overcome the negative effects of immunosuppression. Activation of macrophages, results in increased non-specific phagocytic activity killing pathogens more efficiently and thereby preventing disease outbreaks.

The phenol oxidase system is an important element of the disease resistance of crustaceans. It is, however, of crucial biological significance that this latent defense apparatus is able to identify a real infection and not be switched on by signals other than those unique to pathogens. Crustaceans use LPS and the β-1,3 glucan structure as specific signals to activate the proPhenol oxidase system. The blood of crustaceans contains proteins that specifically bind to β-1,3 glucans. When this protein has reacted with β-1,3 glucan, it can bind to a specific receptor on the haemocytes and induce degranulation and release

of the prophenol oxidase, which can be converted from its proform into an active enzyme by serine proteases. This specific β-1,3 glucan binding protein, whose structure has been revealed, can also act as *opsonin* that stimulates phagocytosis. Phenol oxidase then oxidizes the phenolic group containing amino acids (tyrosine) into semiquinones, which have microbicidal action and these semiquinones are polymerized into melanin.

#### 1.2.1.1. Macrogard

1. ***Product name***

   Macrogard

2. ***Source***

   *Saccharomyces cerevisiae*

3. ***Composition***

   Macrogard consists of 83 % glucose units bound in β-1,3 linkage, 5 % β-1,6 branching points and 6 % β-1,6 linkages and with 5 % of glucose molecules in the non-reducing terminal position.

4. ***Active ingredients***

   β-1,3 glucan

5. ***Mode of action***

   **Fishes**

   In fishes, β-1,3 glucan stimulates the formation of macrophages and elevate their phagocytic and microbicidal activity besides inducing the production of lysozyme, IL-1, TNF and enhancing the production of complement in blood.

   **Prawns**

   In prawns, haemocytes produce more superoxide radicals and hydrogen peroxide. It induces degranulation by haemocytes and activates prophenol oxidase system.

6. ***Mode of application***

   Both immersion treatment and oral administration via diet are recommended.

#### 1.2.1.2. Betafectin

1. ***Product name***

   Betafectin

2. ***Source***

   Genetically modified baker's yeast, *Saccharomyces cerevisiae.*

3. ***Composition***

   β-1,3 glucan with β-1,6 triglycosyl side chains. Concentration not known.

4. ***Active ngredients***

   β-1,3 glucan

5. ***Mode of action***

   The effect of Betafectin on fish and shellfish has apparently not been studied.

**1.2.1.3. Krestin (PSK)**

1. ***Product Name***

   Krestin

2. ***Source***

   β- glucan extracted from mycelia of the basidiomycete, *Coriolus versicolor*.

3. ***Composition***

   A protein linked β-1-3 glucan of molecular weight 100 000 Da.

**1.2.1.4. Lentinan**

1. ***Product name***

   Lentinan

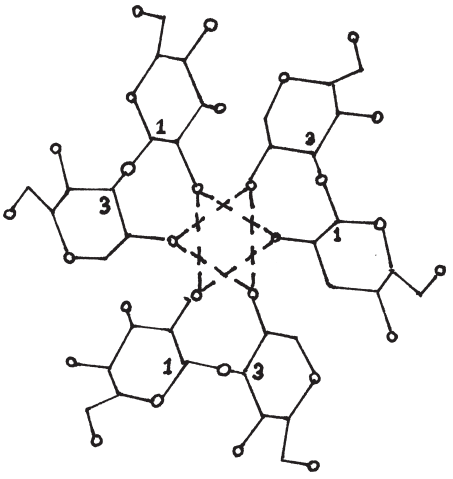
2. ***Source***

   *Lentinus edodes* (a basidiomycete)

3. ***Composition***

   Extracted from basidiomycete, *Lentinus edodes*, the shiitake mushroom. It has two β 1,6 glucose branches for every five β-1,3-glycosyl units in the backbone with molecular weight of $5x10^5$ Da.

***Structure of Lentinan***




4. ***Active ingredients***

   β-1,3-glucan and β-1,6-glucan

5. ***Mode of action***

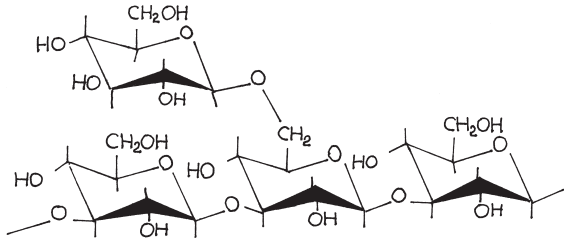
   Increased phagocytic activity of kidney leucocytes in fishes have been noticed on administration with lentinan.

6. ***Mode of application and recommended preparations required for applying in aquatic system***

   The reported application is by injection at a dosage of 2-10 mg/kg body weight in carp (*Cyprinus carpio).*

### 1.2.1.5 Schizophyllan (Sizofiran)

***Structure of Schizophyllan***



1. ***Product name***

   Schizophyllan (Sizofiran)

2. ***Source***

   *Schizophyllum commune* (Fungi)

3. ***Composition***

   In the β-1,3 glucan backbone there are 1,6 glucose branches for every third glucose molecule. Molecular weight is $4.5x10^5$ Da.

4. ***Active ingredients***

   β-1,3-glucan

5. ***Mode of action***

   Induces the formation of cytotoxic macrophages and generates antitumour activity in humans. Increased phagocytic activity of kidney leucocytes and higher complement and lysozyme levels have been reported.

### 1.2.1.6. Scleroglucan

1. ***Product name***

   Scleroglucan

2. ***Source***

   Isolated from the culture medium of basidiomycete *Sclerotium glucanum*.

3. ***Composition***

   In the β-1,3 glucan molecule there are 1,6 glucose branches from every third glucose molecule. Molecular weight is $5.4\ x10^5$ Da.

4. ***Active ingredients***

   β-1,3-glucan

5. ***Mode of action***

   Induces increased disease resistance and the same cellular effect as in carp and yellow tail.

### 1.2.1.7. Vita Stim Taito

1. ***Product name***

   Vita Stim Taito

2. ***Source***

   *Schizophyllum commune* (Fungi)

3. ***Composition***

   Commercial schizophyllan preparation without any details on its composition.

4. ***Active ingredients***

   β-1,3 glucan

5. ***Mode of action***

   Stimulate the immune response of coho salmon by increasing the antibody titre against fish pathogen, *Edwardsiella ictaluri.*

6. ***Mode of application***

   Application is done *via* feed (0.1 % ) for fish weighing > 35g is recommended.

#### 1.2.1.8. SSG

1. ***Product name***

   SSG

2. ***Source***

   From fungus *Sclerotina sclerotiorum*

3. ***Composition***

   A highly branched β-1,3-glucan obtained from the culture fluid of the fungus

4. ***Active ingredients***

   β-1,3 glucan

5. ***Mode of action***

   Augment macrophage function *in vivo*

### 1.2.2. Products from bacteria

#### 1.2.2.1. Biostim

1. ***Product name***

   Biostim

2. ***Source***

   *Klebsiella pneumoniae*

3. ***Composition***

   Capsular glycoprotein

4. ***Mode of action***

   Biostim enhances phagocytic activity and enhances the bactericidal activity of circulating phagocytes in pigs.

   There are no reported studies in aquatic animals.

***5. Mode of application***

Oral application is recommended.

**1.2.2.2. Picibanil (OK-432)**

***1. Product name***

Picibanil (OK-432)

***2. Source***

*Streptococcus pyogenes*

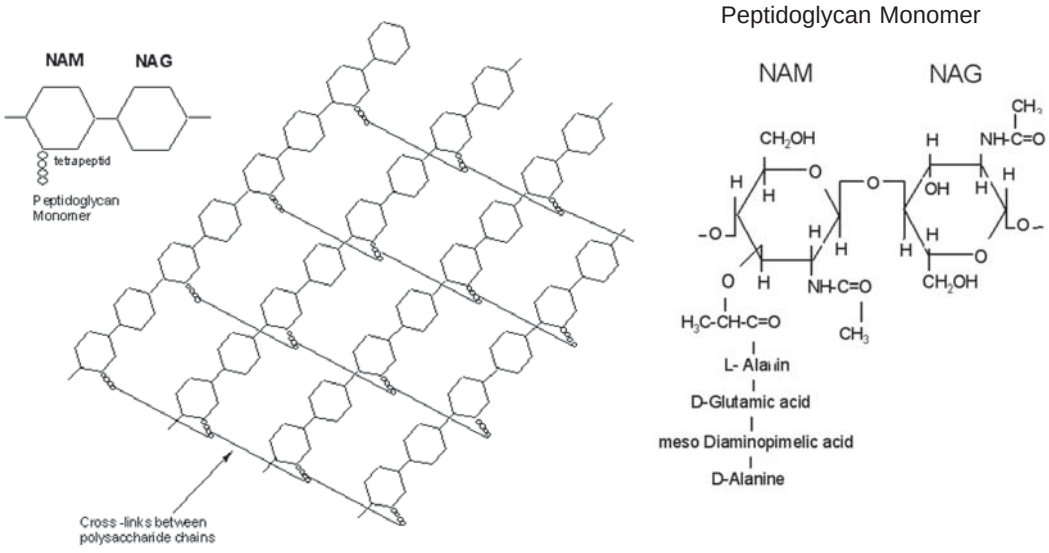
***3. Composition***

It is a lyophilized powder of penicillin treated *Streptococcus pyogenes* biomasss.

***6. Mode of action***

The product activates macrophages and natural killer cells and induces increased production of IL-2, IFN and TFN. No reports of its use in aquatic animals.

**1.2.2.3 Peptidoglycan**




***Structure of peptidoglycan***

***1. Product name***

Peptidoglycan

***2. Source***

*Brevibacterium lactofermentum* or *Bifidobacterium thermophilum.*

***3. Composition***

N-acetyl muramyl L-alanine-D-isoglutamine

***4. Active ingredients***

N-acetyl muramyl L-alanine-D-isoglutamine

***6. Mode of action***

Induced disease resistance in kuruma shrimp against *Vibrio penaeida* and white spot syndrome baculovirus and increases the phagocytic activity of haemocytes in *Penaeus monodon*.

**7. *Mode of application***

Oral via diet

**1.2.2.4. Bacille Calmette Guerin (BCG)**

***1. Product name***

Bacille Calmette Guerin (BCG)

***2. Source***

*Mycobacterium bovis*

***3. Composition***

Cell wall preparation of *Mycobacterium bovis.*

***4. Active ingredients***

Compound of mycolic acid - arabinogalactan - mucopeptide complex.

Mycolic acid is a branched chain β- hydroxy fatty acid connected by an ester linkage to arabino galactan and further connected to the mucopeptide by a glycosidic linkage. The mucopeptide contains alanine, glutamic acid, diaminopimelic acid, N - acetyl glucosamine, and N-glycolyl muramic acid.

***5. Mode of action***

BCG produced increased resistance in rainbow trout to enteric red mouth disease.

***6. Mode of application***

Injection

**1.2.2.5. FCA– Freunds Complete Adjuvant.**

***1. Product name***

FCA– Freunds Complete Adjuvant.

***2. Source***

*Mycobacterium*, *Nocardia* and *Corynebacterium*.

***3. Composition***

FCA consists of cell wall preparations of *Mycobacterium, Nocardia* and *Corynebacterium s*uspended in mineral oil.

4. ***Active ingredients***

A mycolic acid - arabinogalactan - mucopeptide complex

***5. Mode of action***

In fish, increased phagocytic and bactericidal activity is reported. Maximum level of resistance is effected after 30 days of injection.

***6. Mode of application***

Injection

7. ***Impact on the environment and known side effects in animals and human beings if any***

Cause granulomas and open lesions at the injection site and dramatic reduction in growth.

**1.2.2.6. Lipopolysaccharide**

***1. Product name***

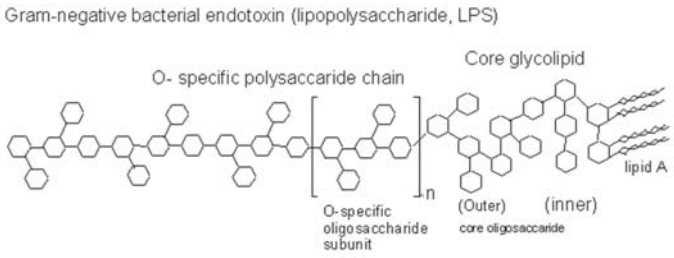
Lipopolysaccharide

***2. Source***

Gram-negative bacteria (cell wall)

***3. Composition***

***Structure of Lipopolysaccharide (LPS)***




The outer portion of the LPS molecule– the O polysaccharide consists of common hexoses such as glucose, galactose, mannose and rhamnose as well as a couple of unique sugars. Between the O polysaccharide and the lipid fraction of the LPS molecule, there is a conservative core polysaccharide, which contains a unique eight carbon sugar (ketodeoxy octonate), seven carbon sugar and glucose, galactose and N-acetyl glucosamine. The lipid complex of LPS is linked to the core polysaccharide and this lipid is composed of phospholipids with saturated fatty acids.

***4. Active ingredients***

It is the lipid moiety in association with the core polysaccharide of LPS that is responsible for the biological activities.

***5. Mode of action***

Diverse non-specific action on the immune system, activating both macrophages and lymphocytes interaction with reporting of increased production of IFNs.

In mammals, macrophages activated by LPS secrete a wide range of molecules that modulate other functions of the immune system such as TNF - a, interleukins IL-1 and IL-6, Prostaglandins (PGF2), free radicals ($O_2^-$) and Hydrogen peroxide. LPS stimulates fish leucocytes *in vitro.* Stimulation of monocytes of channel catfish with LPS resulted in the release of IL - like factors. In salmon, LPS stimulate lymphocyte proliferation. In eels, the injection of LPS from the bacterial pathogen *Edwardsiella tarda* resulted in increased phagocytic activity and disease resistance.

6. ***Impact on the environment and known side effects in animals and human beings, if any***

   Causes very high toxicity in warm blooded animals on administering in higher concentrations. Monocytes of warm-blooded animals have specific LPS binding proteins that the fishes lack.

**1.2.2.7. Muramyl peptides**

1. ***Product name***

   Muramyl peptides

2. ***Source (Chemical/Biological)***

   Mycobacterial cell wall preparation

3. ***Active ingredients***

   N - acetylmuramyl L - alanyl D - isoglutamine (MDP)

4. ***Mode of action***

   In human beings, MDP affects T and B lymphocytes directly and stimulates macrophages directly as well to secrete ILs, TNF, which in turn stimulate the formation of monocytes and granulocytes. Experiments have shown that a MDP derivative injected into rainbow trout increased phagocytic activity, respiratory burst, and migratory activity of kidney leucocytes two days after injection.

5. ***Mode of application and recommended preparations required for applying in aquatic system***

   Injection– 2 mg/kg body weight

**1.2.2.8. Curdlan**

1. ***Product name***

   Curdlan

2. ***Source***

   *Alcaligenes faecalis*

3. ***Composition***

   β-1,3 glucan is extracted from the culture broth of the bacterium *Alcaligenes faecalis*.

The number of branches on the 1,3 glucan backbone is low compared to glucans in fungi. While the primary structure is a long chain, Curdlan forms more complex tertiary structure due to intramolecular and inter-molecular hydrogen bonding.

***4. Mode of application***

Oral application is recommended

## 1.3. Products from plants/algae

### 1.3.1. Glycans

Many different glycans (*i.e.*, Polysaccharides also containing sugars other than glucose) exist in green plants and some are major constituents of cell walls.

***1. Product name***

Arabinogalactan

***2. Source***

From cell cultures of the plant, *Echinacea purpurea.* Molecular weight 75 000 Da.

***3. Composition***

Arabino galactan

***4. Mode of action***

Interacts with mice macrophages and induces a cascade of cellular and biochemical events comparable to those elicited by β (1,3)-D glucans. No information in aquatic animals is available.

### 1.3.2. Laminaran

Laminaran is a β (1,6) branched β (1,3)-D glucan, a major component in sublittoral brown algae, *e.g*., Phaeophyceae, and occurs principally in the Laminarae. Almost all β (1,3)-D glucan display poor water solubility, which makes them less easy to handle than aqueous soluble laminaran. Laminaran obtained from *Laminaria hyperborea* has immunomodulatory effects on anterior kidney macrophages *in vitro* after intravenous adminisration. Intraperitoneal injection of laminaran has also been shown to be preventing mortality caused by *Aeromonas hydrophila* infection in blue gourami, *Trichogaster trichopterus*. Therefore, it is likely that laminaran has the potential to enhance the non-specific defense against infectious disease, either administered perorally or as a feed additive, or intraperitoneally by injection.

### 1.3.3. Glycyrrhizin

Glycyrrhizin is a glycosylated saponin, containing one molecule of glycyrretininc acid, which has anti-inflammatory activities and anti-tumor activities mediated by its immunomodulatory activities. Yellow tail treated orally with glycyrrhizin showed

increased protection against *Edwardsiella seriola* infection, although lysozyme activity of blood and phagocytic activities of macrophages were not enhanced.

## 1.4. Products from animals

### 1.4.1. Chitin

***1. Product name***

Chitin

***2. Source***

Crustacean shell

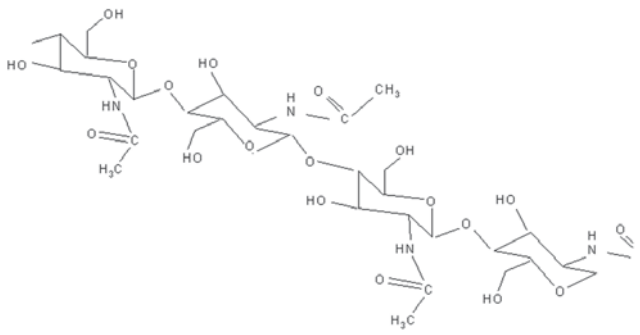
***3. Composition***

Polymer of N-acetyl Glucosamine

***4. Active ingredients***

Chitin.

***Structure of chitin***



***5. Mode of action***

Acts like an unspecific irritant of the leucocytes by interacting with specific receptors. Increases the phagocytic activity and respiratory burst of kidney leucocytes 5 to 7 days later.

***6. Mode of application and recommended preparations required for applying in aquatic system***

Injection at a dose of 100 mg/kg body weight is recommended. Chitin can be incorporated in diet also.

### 1.4. 2. Peptides from animal extracts

It has been demonstrated that a peptide from fish byproduct enhances the non-specific defense and immune response of fish.

#### 1.4.2.1. Peptide from Cod

***1. Product name***

Peptide cod: Molecular weight <3000 Da.

***2. Source***

Enzymatic hydrolysate of cod protein

***3. Mode of action***

Activates head kidney leucocytes of salmon.

#### 1.4.2.2. Thymopeptin

1. ***Product name***

   Thymopeptin

2. ***Source***

   Thymus gland

3. ***Active ingredients***

   Peptide

4. ***Mode of action***

   Augments the functions of T lymphocytes, but does not induce the formation of new cells.

#### 1.4.2.3. Thymosin (28 aminoacids)

1. ***Product name***

   Thymosin (28 aminoacids)

2. ***Source***

   Thymus gland

3. ***Active ingredients***

   Peptide

4. ***Mode of action***

   Enhances the production of IL and causes receptors for IL to be displayed on the T lymphocytes.

### 1.4.3. Ete and Hde

An extract from marine tunicate, *Ecteinascidia turbinate* (Ete) and glucoprotein fraction of water extract (Hde) from Abalone, *Halotis discus* bannai, enhanced the killing of tumor cells *in vitro* and inhibited tumor growth *in vivo*. Eel injected with Ete showed enhanced phagocytosis and increased survival following *A. hydrophila* challenge. Rainbow trout injected with Hde also showed enhanced phagocytic and NK cell activities and showed increased survival against *V. anguillarum* infection.

## 1.5. Synthetic compounds

### 1.5.1. Bestatin

1. ***Product name***

   Bestatin

2. ***Source***

   First isolated from the culture filtrate of *Streptomyces abirorectuli* and later synthesized.

***3. Composition***

Dipeptide

***4. Mode of action***

In human beings it activates macrophages and natural killer cell activity.

No reports from aquatic animals.

**1.5.2. Lipopeptides**

**1.5.2.1. FK - 156**

***1. Product name***

FK - 156

***2. Source***

Originally isolated from the culture filtrate of *Saccharomyces olivaceogriseus* and later synthesized.

***3. Composition***

A lactyl tetrapeptide

***4. Mode of action***

Enhances resistance to microbial infections in warm-blooded animals.

**1.5.2.2. FK - 565**

***1. Product name***

FK - 565

***2. Source***

Chemically synthesized as the analog of FK - 156.

***3. Mode of action***

Increased phagocytic and bactericidal activity one day after injection. Activates leucocytes *in vitro* (measured by respiratory burst and spreading activity).

**1.5.2.3. Levamisole**

***1. Product name***

Levamisole

***2. Source***

Synthetic compound

***3. Composition***

2,3,5,6- tetrahydro - 6 - phenylimidazo (2,1-***b***) thiazole

4. ***Basic scientific data on field trials***

   Rainbow trout fed on levamisole at 5 mg/kg body weight induced high degree of protection against *Vibrio anguillarum* challenge.

5. ***Mode of action***

   Increases phagocytic activity of kidney leucocytes five day post administration. Increased complement and bactericidal activity.

6. ***Mode of application and recommended preparations required for applying in aquatic system***

   Injection - 0.5mg/kg body weight in trout

   Injection - 5-10 mg, 3 times at 3 days interval in carp

   Orally - Levamisole incorporated diet for 15 days at 3 days interval

   Bath treatment - Rainbow trout given bath treatment (5µg/ml) showed higher disease resistance

7. ***Impact on the environment and known side effects in animals and human beings, if any***

   Higher dose may cause immunosupression.

## 1.6. Vitamins

Vitamins are organic nutrients that are required in small quantities for a variety of biochemical functions and which generally cannot be synthesized by the body and must therefore be supplied from diet. Of the various vitamins, Vitamin C and Vitamin E are the most important nutrients that influence immune system.

### 1.6.1. Vitamin C

Vitamin C is a water-soluble antioxidant that maintains many metal co-factors in the reduced state. Ascorbic acid deficiency increases disease susceptibility in different species such as rainbow trout and Atlantic salmon and affects the immune system. Several effects of vitamin C deficiencies have been reported, such as reduction of antibody production and complement activity in Atlantic salmon or reduction of macrophage in Channel catfish. Mortality rates of fish experimentally infected with *Edwardsiella ictaluri* causing enteric septicemia in channel catfish, decreases with increase in dietary ascorbic acid doses, ranging from 100 % for fish fed with the ascorbic acid deficient diet to 15 % for fish fed with 300 mg ascorbic/kg diet. There were no differences in antibody production, complement activity or phagocytic activities among fish fed diets containing 30-300 mg ascorbic acid/kg of diet. However, the dose level of 3000 mg ascorbic acid/kg diet significantly enhanced antibody production and complement activity. High levels of dietary vitamin C increases resistance to *Vibrio anguillarum* and *Icthyophtirius multifilis* in rainbow trout. These results show that fishes fed with high doses (more than 1000 mg/kg diet) of vitamin C have protective immune responses.

### 1.6.2. Vitamin E

Vitamin E (Tocopherol) is a naturally occurring antioxidant. It is essential for normal reproduction in many animals, hence known as antisterility vitamin. Vitamin E also enhances both humoral and cellular defenses in mammals. Catfish fed with high doses of vitamin E had increased phagocytic indices and superoxide anion production by leucocytes. Vitamin E supplementation seems to enhance antibody production against *Yersinia ruckeri* in rainbow trout, serum complement function in channel catfish, ability of serum to opsonize bacteria in Atlantic salmon and the activity of alternate complement pathway in gilthead sea bream.

## 1.7. Hormones, cytokines and others

### 1.7.1. Hormones

The relationship between neuroendocrine regulation and the immune system has recently become the subject of intense investigation. It is also known that growth hormone (GH) and prolactin (PRL) directly affect immune competent cells *i.e* macrophages, lymphocytes and NK cells. In fish, exogenous GH has mitogenic activity on lymphocytes and activates NK cells. It has been reported that exogenous GH given to rainbow trout increased the production of superoxide anion in leucocytes. GH receptors are present in monocytes and lymphocytes of gilthead Sea bream, *Sparus aurata*.

Prolactin (PRL) also has immunostimulatory activities. The addition of homogenous PRL to chum salmon, *Oncorynchus keta* induced lymphocytes mitogenic responses and PRL increased the production of superoxide anion by leucocytes in rainbow trout.

Melanin stimulating hormone (MSH) and melanin concentrating hormone (MCH) stimulate phagocytosis of head kidney leucocytes of rainbow trout *in vitro.*

### 1.7.2. Cytokines

The molecules involved in the transmission of signals between leukocytes, such as IFN, IL, TNF, monocyte chemotactic factor and colony stimulating factors have dominated research on immunotherapy in humans during the last few years. Such cytokines, which have their counterparts in fish, are produced by only one cell type and the prospects of using such products in aquaculture are not realistic at present due to high costs involved. Although it is a fascinating option to use pure cytokines to enhance immunity, it seems to be very risky if the concentrations reach non-physiological levels. For example concentrations outside the range occur when the immune system is challenged by natural infections and by immunostimulants that mimic such infections.

### 1.7.3. Lactoferrin

Lactoferrin, which consist of a single polypeptide chain with molecular weight of about 87 000 Da and possessing 2 Fe binding sites per molecule, is widely distributed in physiological fluids of mammals. Rainbow trout orally treated with bovine lactoferrin showed enhanced phagocytic activities and the production of superoxide anion by macrophages and demonstrated high resistance (40 fold increase in $LD_{50}$) to *V. anguillarum*

infection, but marginal increase in resistance (4 fold increase in $LD_{50}$) to *Streptococcus* sp. Lactoferrin can activate macrophages *in vitro*. Red sea bream, orally administrated bovine lactoferrin showed increased numbers of granulocytes in the blood, elevated secretion of body mucus and resistance to cryptocaryon irritants infections.

### 1.7.4. EF 203

EF 203 is a fermented product of chicken egg. The oral administration of EF 203 to rainbow trout stimulates the activity of leukocytes such as phagocytosis and chemilumenescence and increases protection against *Streptococcus* infection. Rainbow trout, *O. mykiss* when vaccinated after being treated with EF 203 showed higher phagocytic activities as compared with the non-vaccinated fish. The nitro blue tetrazolium (NBT) reduction increased in kidney leococytes of vaccinated and EF 203 treated fish groups.

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CHAPTER 11

# Diagnostics in Aquaculture*

## 1. Introduction

Achieving nutritional security in terms of valuable protein for the growing population of the Indian sub-continent is a major challenge in the new millennium. In this endeavour 'aqua farming' is to play a major role, as the growth in agriculture and animal husbandry has been slowing down. It is estimated that about 5 million tonnes of aquatic animal products can be produced annually through aquaculture in India. However, like in other animal rearing systems, diseases form the major hindrance in achieving the target.

Disease problem was not a major deterrent when aquaculture activities were extensive in nature, as in the case of traditional shrimp culture systems of Kerala, Goa and West Bengal. Creation of intensive rearing systems, aiming at higher production and increased profits, without proper planning and management has invited severe health problems and diseases. Unlike the land-based animal rearing systems, where the diseased animals can be identified and treated individually, the scope for disease control in aquaculture through detection and treatment is only of limited value, mainly due to co-existence of the pathogen in the aquatic rearing system. In the grow-out system, fish is constantly bathed in potential pathogens, *viz.*, parasites, bacteria, fungi and viruses. Separating the infected or diseased animals from the population and subjecting them to treatment is impractical, and if at all possible, it is not an economically viable measure. Hence disease treatment becomes a difficult proposition in aquaculture and disease prevention is a natural choice.

The growth, profitability and sustainability of aquaculture primarily depends on successful prevention or control of disease outbreaks. Unlike land-based husbandry systems, disease problems in aqua farming are complicated due to the three-dimensional nature of the system where the dynamic interaction of biotic fauna comprising the host and opportunistic pathogens and abiotic factors exists. The coexistence of host and pathogen in an aquaculture-rearing environment makes it all the more difficult to control/treat the disease. Disease prevention in aquaculture is not merely a case of dealing with the pathogen and its elimination, but needs to be dealt with in a broader perspective. It requires a holistic approach, the focus being on the health of the animal rather than treatment of the disease. Therefore, different components *viz.*, animal quarantine, screening of broodstock and larvae/fingerlings, SPF broodstock, pond and water quality management, good nutrition, disease monitoring/ pathogen watch are integrated. The management practice that is designed to prevent the occurrence of diseases in aquaculture system is termed as fish health management. Implementation of major components of aquatic animal health management, *viz.*, animal quarantine, screening of broodstock and larvae and pathogen watch requires sensitive and

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specific techniques for detection of pathogens. In recent years, a number of highly sensitive, specific and rapid diagnostic techniques have become available for aquatic animal health management. These techniques can be effectively used to screen apparently healthy animals to ensure that they are not harbouring the potential pathogens asymptomatically. Further, diagnostics help in the detection of pathogens causing ill health or abnormalities, in order to draw proper disease control measures. The failure of accurate diagnosis of pathogens can lead to faulty treatment resulting in multiple problems like indiscriminate use of chemicals and drugs, consequent drug resistance, large-scale mortality leading to crop failure and economic loss. The occurrence of disease can be prevented *by detection and avoidance of the pathogen*. Timely and early use of proper diagnostics can be used as an effective tool of health care management.

Over a period of time, an array of diagnostic tools has been developed for aquaculture use, mostly adapted from human and veterinary medicine. These include the first generation tools such as direct microscopy and histopathology, electron microscopy and conventional microbiology, followed by the second generation diagnostics: bioassay, tissue culture and immunoassays. The recent ones are the third generation DNA-based diagnostic tools, such as polymerase chain reaction (PCR) and DNA-hybridisation. But the choice of the diagnostic tools depends on the specific needs of the farmer or the researcher with reference to the specific nature and situation. In general, the efficiency and acceptability of a diagnostic tool in routine use depends on the specificity, speed, reproducibility and simplicity of the method. A single test or a combination of tests need to be performed at the right time, depending on the problem. The success depends on the *right choice and use of the diagnostics* by the aqua culturists to make rapid, prudent and cost-effective management decisions. *Diagnostic tools are not the solution, but the means for an effective aquaculture health management.*

Compared to veterinary medicine, the diagnosis of disease in fish is developing slowly. This is mainly due to the complexity of the aquaculture system, where a close observation on the affected animals is not possible. Further, other than the pathogens, the biotic and abiotic factors can have a direct involvement in the disease development process. Another constraint on successful diagnosis is the rapid post-mortem changes in fish. In crustaceans, autolysis of the midgut gland hepatopancreas or the liver starts as early as 10 minutes after death.

Typically, the diagnostic techniques used in aquaculture can be divided into three levels, first level (farm/hatchery level) or basic, second level (requiring moderate laboratory facilities), and third level or advanced (requiring modern equipment and expertise in molecular biology). One has to select the level of diagnostic technique depending on the purpose of diagnosis and the available laboratory facilities.

The active elements necessary to achieve quality testing have been listed in the Diagnostic Manual for Aquatic Animal Diseases, published by the Office International Des Epizooties (OIE, 2000, website: www.oie.int) and the Asia Diagnostic Guide (FAO, 2001). The OIE is an inter-governmental organization established to promote world animal health, and brought out norms to provide a uniform approach to the diagnosis of the diseases listed in the OIE

International Aquatic Animal Health Code in the form of a *Diagnostic Manual for Aquatic Animal Diseases*. This manual is the fundamental document describing methods that can be applied to the OIE notifiable and other significant aquatic animal diseases. Later, the Food and Agricultural Organization of the United Nations (FAO) and the Network of Aquaculture Centres in Asia-Pacific (NACA, website: www.enaca.org) brought out the *Asia Diagnostic Guide* (FAO Fisheries Technical Paper 402/2, 2001) in accordance with the OIE manual, and WTO's Sanitary and Phytosanitary Agreement (SPS) and support of relevant provisions in the FAO's Code of Conduct for Responsible Fisheries.

## 2. First level or basic diagnosis

First level or basic diagnosis is done with the help of information made available on the history, behavior and gross signs from hatchery-produced seeds/farmed animals. Once the infection or disease is suspected, the next step is to draw a diagnostic procedure, to fix the root cause of the problem. The diagnostic procedure may include a single diagnostic test or a combination of tests, depending on the specific situation. In the case of routine pathogen watch or health monitoring, a set of selected diagnostic tests is performed to cover the potential pathogens. The approach generally followed is location-specific and problem-specific, where the first consideration is the availability of the diagnostic facility and expertise. There is no hard and fast method that can be applied for all cases.

### *2.1. History or background information*

History or background information of the disease at the hatchery/farm facility or in the region, source of seed, type of feed used, environmental conditions with special reference to water quality can provide the first level of data in the fish disease diagnostic process. Proper records have to be maintained of all the relevant information pertaining to seed production/farming practices.

#### *2.1.1. Water-soil quality*

Water provides a dynamic support for the existence of aquatic animals from where they draw nutrients and carry out all their metabolic and catabolic activities. Poor water quality can cause diseases. Whenever fishes are sick, water quality must be first tested. The important water quality parameters are: dissolved oxygen, ammonia, nitrite, pH, temperature, turbidity, alkalinity and hardness. Oxygen depletion or build up of ammonia can result in fish kills, many times more than the mortality due to pathogens. The optimum ranges of water quality parameters for fish vary according to species, age, geographical locations and the type of rearing conditions such as stocking density and feeding conditions. Water quality is very much influenced by soil quality and the most important parameters to be monitored are pH, E*h*, cationic exchange capacity and total organic carbon.

### *2.2. Behaviour*

Normal behaviour pattern of the fish has to be understood and recorded. Deviation from normal behaviour could be expected whenever they are exposed to stress factors

or infective agents. Changes in normal behaviour of fishes can offer hints regarding the problem.

### *2.2.1. Finfishes*

Clinically important behaviour patterns of fishes are: lethargy- a decrease or complete lack of normal activity; anorexia- loss of normal feeding activity; colour change- indicated by the fish appearing lighter or darker than normal or a change in the intensity of normal colour. Sick fish often appear darker than normal. Swimming pattern of the diseased fish is different from the normal exhibiting drifting, hurdling, whirling, hovering, flashing, rolling, piping, *etc*.

### *2.2.2. Shrimps*

Lethargy, anorexia, fouling the surface, detritus in gills, colour change and abnormal swimming patterns are the common behavioral signs among the farmed shrimps.

Behavior is only an indication and it is important to recognize the underlying reason of abnormal behavior. A clear understanding of what each behavior means is a good adjunct and can be useful in the process of disease diagnosis.

Incidence of mortalities in the rearing facility has to be observed closely. The level and extent of mortality should be recorded. Increasing rate of mortality is indicative of infectious disease agents, warranting interventions in the form of disease investigation and disease management.

### *2.3. Gross external features*

*Finfishes:* Physical observations like lesions, haemorrhage, colour changes, fouling, bulging and corneal eye opacity, excess mucous secretions are important gross external signs among finfishes.

*Shrimps*: Necrotic lesions over the body surface, melanized spots, erosion of setae on uropod.

## 3. Second level or laboratory-based diagnosis

The first level or basic diagnosis can only act as pointers or provide hint about the problem. It is not pathognomonic. Hence, to have a dependable and reliable diagnosis, specific identification of pathogens using relevant laboratory methods is imperative.

### *Laboratory facilities*

A properly designed and well-equipped laboratory is pre-requisite to conduct second level and advanced diagnostic tests. The laboratory must have adequate dust proof space with proper ventilation, lighting, reliable electric supply, well maintained equipment and operational materials, sufficient storage facilities, archiving facilities along with access to necessary literature reference.

### *Personnel*

A diagnostic laboratory should have qualified and experienced staff at managerial, supervisory and technical levels to take up tasks in all fields of fish disease diagnostics. Proper professional support can produce consistent test results of high quality.

***Selection of diagnostic tests, protocol and validation***

The methods to be used in the laboratory should be selected on the basis of international acceptance, scientific screening, performance factors such as sensitivity, specificity and reproducibility, simplicity, cost-effectiveness, user expectation and safety. Validation of the tests has to be conducted from time to time and the tests must be subjected to comparative evaluation with other methods and evaluative studies with other laboratories through exchange of samples and test results. Experimental challenge and epidemiological studies may also be included as required. Tests should be conducted on the basis of detailed test protocols with guidelines about the test materials and controls, detection limits, criteria for acceptance or rejection of the test results and methods of diagnostic interpretation. A laboratory should keep the security, integrity and irretrievability of test records and all relevant data for a specified period of time (not less than 5 years after the completion of test). A quality management programme within the laboratory should be in place to ensure the reliability of the test results, corrective and preventive action if needed, with scope for continuous improvement by interacting with local and international organizations such as OIE, FAO, NACA, *etc*.

## 3.1. Laboratory methods in fish disease diagnosis

### *3.1.1. Fish sampling for laboratory observation*

From fish exhibiting clinical signs, collect ten fishes (minimum four) representative of the disease problem for diagnosis. The affected or moribund fish can be collected alive and transported to the laboratory. Alternatively, required tissue samples, organ or fluids can be collected from the rearing site as per test requirement (in proper fixatives or in a sterile container in ice) and used for processing at the earliest. *Dead or frozen fish cannot be taken for any diagnosis.*

While samples of fish from a population without any clinical manifestation are to be collected randomly to assess the health status or prevalence of infection, the collection should contain required number of specimens drawn on the basis of a statistically proven model based on guidelines provided for sample size (see Table on page 172).

### *3.1.2. Clinical work-up*

#### *3.1.2.1. Skin and gill biopsy*

***Finfish:*** Once the history, behavior and gross external features have been recorded, biopsies of skin and gills should be conducted for external pathogens. Fishes larger than 2 cm can be used for skin biopsy and those larger than 5 cm can be used for gill biopsy. Skin and gill smears can be collected from live fish using glass slides. Then a drop of water is added to the smear and covered with a cover slip and examined for parasites using a compound microscope under magnifications ranging from 40X to 400X.

Skin and gill biopsies are useful tools for diagnosing diseases in fish, as skin and gills are the primary target organs for a number of pathogens. Larger parasites such as trematodes and crustaceans can be seen with the naked eyes or with the help of a hand

lens. Most protozoans and fungal hyphae are visible at lower magnifications of 40X and 100X, while higher magnification of 400x and 1000X is required for definitive identification of protozoans and bacteria.

***Crustaceans:*** In the case of cultivable crustaceans such as shrimp, prawns and crabs, microscopic examination of gills, appendages and larval stages observed under bright field illumination or phase contrast, under a magnification of 40X, 100X and 400X reveals the parasites and fungal hyphae.

Microscopic examination of squash preparations of hepatopancreas, intestine and faecal matter stained with malachite green (0.01 %) or eosin can detect the presence of protozoan parasites and viral occlusions. Impression smears of hepatopancreas or suspected tissues fixed with methanol and stained Giemsa or acid fast stain can also be used for microscopic examination.

**Table: Sample sizes to detect at least one infected host in a population of a given size, at a given prevalence of infection. Assumptions of 2 % and 5 % prevalences are most commonly used for surveillance of presumed exotic pathogens, with a 95 % confidence limit (Lightner, 1996)**

| Population Size | Prevalence (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 10.0 |
| 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 | 71 | 57 | 27 |
| 10000 | 579 | 292 | 146 | 96 | 72 | 29 | 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 |

### *3.1.2.2. Collection of blood sample*

***Finfish:*** Blood samples are required to examine the presence of haemo parasites such as trypanosomes and for microbiological examinations to check bacteremia. The caudal vein is the ideal site for blood sampling. Fish should be adequately sedated with MS-222 and 2 ml or 5 ml syringe fitted with 20 or 21 gauge needle, both rinsed with anticoagulant heparin is used to collect blood. Another site of blood sampling is the heart. The only

way of blood sampling from small fishes is to cut the caudal peduncle and collect the blood directly into a heparinised tube.

***Crustaceans:*** Haemolymph samples for microbiological and microscopic studies can be collected by cardiac puncture or through ventral sinus with tuberculin syringe fitted with 26, 27 or 28 gauge needle from a live or moribund animal. An anticoagulant such as 3-8 % tri-sodium citrate is used to prevent clotting. In the case of crabs, to collect haemolymph, one of the chelae is broken and the oozing haemolypmh is collected directly into a glass tube.

#### *3.1.2.3. Examination of internal organs*

***Finfishes:*** Further dissection has to be performed aseptically to collect tissue samples for microbiology and histopathology and also for the assessment of gross internal features. Bacteriological sampling of the internal organs has to be conducted soon after the opening of body cavity. Kidney is the preferred organ for the isolation of bacterial pathogens.

Six tissues routinely used for detailed examination using wet mount preparation and histopathology are the gills, liver, heart, spleen, kidney and gastrointestinal tract. Other tissues such as eye, swim bladder and brain are also used for investigation. Squash preparations using these tissues are useful for locating microsporidians, myxosporidians and mycobacteria.

***Crustaceans:*** The important tissues which are used for routine observation are gills, heart, hepatopancreas, lymphoid organ and gut and intestine.

## 4. Histological methods

Histology, the study of the micro-anatomy of specific tissue has been successfully employed as a diagnostic tool in fish diseases, as is the case in medical and veterinary sciences. Knowledge about the pathological manifestations plays a very important role in the diagnosis of diseases. Many of the recognised diseases of penaeid shrimp, especially viral diseases, were first recognised and diagnosed by routine histological procedures. Even today, pathological manifestations based on histological sections stained with hematoxylin and eosin form the most important tool in fish disease diagnosis.

### *4.1. Collection and preparation of materials/samples*

For histological studies, the tissue samples should be removed from living or from anaesthetised animal by biopsy as quickly as possible. Delay will lead to warming up of tissues resulting in autolysis. Moribund fish, discoloured and those displaying abnormal behaviour, are the best samples for diagnostic purposes, except in the case of intentional random sampling for estimation of disease prevalence. Complete history of the specimens such as gross observation, species, age, weight, source, etc. and any other pertinent information helpful in diagnosis should be recorded.

### *4.2. Fixation of samples*

The term 'fixation' means immobilization. This is one of the crucial steps in histological procedure. The main objective of fixation is to preserve the cellular configuration of the

tissue by preventing self-destruction of tissues through autolysis and bacterial degradation (putrefaction) besides denaturation of the proteins in the tissues. The tissue is fixed after being taken out from the specimens, to avoid post-mortem changes. Fixation of fish tissues can be done in many ways. The whole specimen can be fixed live by immersion or injection of the fixative into vital areas before immersion with proper fixative. Generally, 5-10 times the volume of fixatives should be used for each specimen. Various fixatives have been used for the preservation of fish with varying success. These are **simple** fixatives (*e.g.* formalin, methanol, ethanol, etc.) or **compound** fixatives (Buffered formalin, Bouin's fluid, Davidson's fixatives) in which mixtures of several fixing agents are used in liquid form. Ten percent neutral buffered formalin (NBF) is the commonly used fixative and a 24 h fixation is sufficient in most situations. The tissue can be left in the NBF for long periods without excessive hardening or damage. While Bouin's fixative is specifically used in fixation of finfish tissues, especially for eyes, gill, skin, reproductive tissues and small fishes. Most routine histological studies of crustaceans employ Davidson's Alcohol Formalin Acetic acid (AFA) as the fixative. However, NBF is also recommended.

***Composition of Neutral Buffered Formalin (NBF)***

| | |
|---|---|
| Formalin (40 % formaldehyde) | 100 ml |
| Distilled water | 900 ml |
| Sodium dihydrogen orthophosphate | 4 g |
| Di-sodium hydrogen orthophosphate | 6 g |

- Fixation time 24 h to indefinite.

***Composition of Bouin's Fluid***

| | |
|---|---|
| Picric acid saturated aquous solution | 75 ml |
| Formalin (40 % formaldehyde) | 25 ml |
| Glacial acetic acid | 5 ml |

Fixation time 24-48 hours at room temperature. Then transfer to 70 % ethyl alcohol for storage.

***Composition of Davidson's Alcohol Formalin Acetic Acid fixative (AFA)***

| | |
|---|---|
| 95 % Ethyl alcohol | 30 ml |
| Formalin | 20 ml |
| Glacial Acetic acid | 11.5 ml |
| Distilled Water | 33.5 ml |

Fixation time 24-48 h at room temperature. Then transfer to 70 % ethyl alcohol for storage.

Out of these three fixatives, Davidson's fixative is the best for shrimp histology. Larvae and early post-larvae can be directly immersed in the fixative. Juveniles and adult shrimps should be injected with 1-10 ml (depending on the size of shrimp) of fixative into

hepatopancreas, region anterior to hepatopancreas, anterior abdominal and posterior abdominal regions. A large share of fixatives should be injected into the cephalothorasic region and posterior abdominal region. The amount of fixative can vary, approximately 5-10 % of body weight. After the injection, cut open the cuticle from sixth abdominal segment to the rostrum with a sharp scissor, without damaging the internal organs. The specimens should be immersed in 5-10 volumes of fixative (*i.e.* tissue of 1 ml volume requires 10 ml fixative) for 24 h. For large animals, fixation can be done even up to 48 h. After that, the specimen is transferred to 70 % ethyl alcohol for storage.

### 4.3. Decalcification

A specimen may contain a mixture of hard and soft tissues. The soft tissues can be processed for histological examination without any special treatment. However, hard-calcified tissues such as cuticle may require special treatment like decalcification. This process will soften the calcified tissues by removing calcium ions from bony components, sufficient to allow smooth sectioning. Tissues fixed in Davidson's fixative or NBF have to be placed in decalcifying solution for 24-72 hours depending upon the nature and size of the tissues. After proper decalcification, wash the tissue in 70 % ethyl alcohol 2-3 times and store in fresh 70 % ethyl alcohol.

**Decalcifying solution**

| | |
|---|---|
| 70 % ethyl alcohol | 98 ml |
| Conc. nitric acid | 2 ml |

### *4.4. Paraffin-wax processing and embedding*

Further processing of the fixed tissue involves dehydration through ascending grades of alcohol (or cellosolve, dioxane, isoprophyl alcohol, etc.), clearing of tissue using a paraffin-miscible solvent such as xylene, chloroform or methyl benzoate and finally impregnation/ infiltration with paraffin wax and embedding (blocking).

#### 4.4.1. Dehydration

In order to infiltrate with paraffin wax, it is first necessary to remove all water from the fixed tissues by dehydration. Dehydration is a process of gradual or step-wise replacement of water by graded dehydrating agents and it is usual to begin with 50-70 % ethyl alcohol, through progressive higher grades of alcohol to saturate the tissue with absolute alcohol to complete the dehydration, as shown below:

| | |
|---|---|
| 50 % alcohol | 1h |
| 70 % alcohol | 1h |
| 90 % alcohol | 1h |
| 100 % alcohol | 30min. X 2 |

However, the duration of treatment in graded alcohol will depend on the size and type of tissue. For tissues not more than 7-10 mm thickness, 70 %, 90 % and 100 % alcohols, and 3 changes for 1-2 hours each are sufficient.

### *4.4.2. Clearing*

As alcohol is not miscible with paraffin wax, it is first necessary to treat the tissue with an agent, which is miscible with both the substances. There are several such reagents in general use of which xylene (or chloroform, toluene, benzene, methyl benzoate, clove oil, cedar wood oil, etc.) is the most favoured. The optimum time, for which the tissue should be kept in a clearing agent, is indicated by the shine or transparency of the tissue (1-2 h).

| | |
|---|---|
| Absolute alcohol + Xylene (1 : 1) | 1h |
| Xylene | 1h X 2 |

### *4.4.3. Infiltration/Impregnation*

The aim of impregnation is to make the tissue firm for the purpose of sectioning with microtome. A low melting point paraffin wax (MP 58-60°C) is used.

*Paraffin cold impregnation*

Xylene and paraffin shavings (1 : 1) 1h

*Hot impregnation*

Transfer the tissue in the cavity blocks or other small tray containing molten paraffin kept at 58-60 °C.

Infiltration time depends on the size and nature of the tissues.

### *4.4.4. Embedding*

The method of embedding or reinforcement of tissue is done using paraffin wax (or celloidin, gelatin, etc.). After proper paraffin infiltration, the tissues can be transferred to appropriate blocks (depending on the size of the tissues) containing molten paraffin. A histoembedder can be used for impregnation in molten paraffin wax, dispensing molten wax for block preparation. Extreme care should be taken to get the correct orientation of the tissue and to avoid air bubbles. Allow the paraffin to solidify and remove the paraffin block containing tissue.

### *4.4.5. Labelling and storage*

Labels with concise information on a small paper in lead pencil is generally inserted on one side of the block during casting. Store the blocks in thick ziplock polybags or wooden boxes with cloth lining or alternatively in a mixture of equal volume of 70 % alcohol and glycerine in well stoppered bottles.

### *4.5. Sectioning*

Sections of the tissues can be taken using a microtome. Before sectioning, the tissue-embedded paraffin blocks should be trimmed to suitable size. Care should be taken to see the proper orientation of the tissue. Fix the trimmed block on to a holder and take the sections in the form of a ribbon of appropriate thickness. Sections of 5-7 µm thickness are good for routine histopathological studies. Two main features govern satisfactory

sectioning of tissues; a clean and sharp knife and reduction in temperature of the block by keeping in a freezer for a few hours to increase its hardness.

The resulting ribbons containing tissue sections can be cut into smaller pieces, put on a clean glass slide, which is coated with egg albumin. One slide can hold one or more ribbons according to the size of the tissue or the width of the ribbon. Proper spreading of the ribbon can be done in two ways.

1. Small pieces of ribbon can be put in a warm water bath (or a tissue floating bath). When the ribbon gets spread due to the high temperature of water, put a clean albumin coated slide underneath the ribbon and just lift the slide in such a way that the ribbon sticks to the surface of the slide. Drain the water and keep the slide in a slanting position on a slide rack free from dust.
2. Cut the ribbon into small pieces; keep one or more ribbons over the slide coated with adhesive, put a few drops of water on the slide so as to float the ribbon on the water surface. Place the slide on a slide-warming table or pass it over a flame of a spirit lamp. Two needles can be used to spread the ribbon to the maximum, drain the water and keep the slide in a slanting position.

Whichever method is followed, care should be taken to avoid wrinkles in the section. Improper spreading will interfere with staining and also microscopic observation. After proper drying, the slides can be kept in a dust-proof box for some time for adequate adhesion. These slides can also be stored indefinitely.

### *4.6. Staining*

Biological stains have been used to visualize and identify tissues and cell components. Before the tissue sections are subjected to staining, the sections should be deparaffinized thoroughly and dehydrated. Hematoxylin and eosin (H & E) staining can be employed for routine histological preparation and this is the best method for histological diagnosis of viral diseases. Steps involved in staining with H & E are as follows.

***Harris' haematoxylin***

| | |
|---|---|
| Haematoxylin crystal | 2.5.0 g |
| 100 % alcohol | 50.0 ml |
| Ammonium/potassium alum | 50.0 g |
| Distilled water | 500 ml |
| Mercuric oxide | 1.5 g |
| Glacial acetic acid (after cooling) | 20 ml |

Dissolve the haematoxylin in absolute alcohol; add the alum previously dissolved in hot distilled water. Heat the mixture to boiling point and add the mercuric oxide, cool rapidly and filter. This stain is ready for use when cool. Staining time is 2-3 minutes.

***Eosin:*** 1 % aqueous eosin or alcoholic eosin

**Procedure**

| | |
|---|---|
| De-paraffinise the slide in xylene | 1h X 2 |
| Absolute alcohol | 15 min X 2 |
| 90 % alcohol | 15 min |
| 70 % alcohol | 15 min |
| 50 % alcohol | 15 min |
| Water | 10 min X 2 |
| Stain in hematoxylin | 2-5 min |
| Wash in water | adequately |
| De-stain in acid alcohol (if needed) | adequately |
| Wash in tap water | 1 min |
| 50 % alcohol | 30 min |
| 70 % alcohol | 15 min X 2 |
| Stain in Eosin | 1 min |
| 90 % alcohol | 15 min X 2 |
| Absolute alcohol | 20 min X 2 |
| Alcohol + Xylene (1 : 1) | 15 min |
| Xylene | 30 min X 2 |

Mount in DPX and label

Observe under microscope.

## 5. Microbiological methods

A number of bacterial genera are associated with shrimp and shrimp culture ponds. The heterotrophic bacteria that are associated with shrimp and its environment belong to various genera such as *Vibrio, Pseudomonas, Flavobacterium, Aeromonas, Alkaligens, Acinetobacter, Bacillus, Micrococcus, etc.* The *Vibrio* and *Pseudomonas* most often predominate. In addition, mycobacteria, rickettsia, clamydia and mycoplasmas are also occasionally reported to be associated with shrimp disease. Majority of the bacteria that are reported to be associated with shrimp disease are opportunistic pathogens. *Vibrio* spp. are the most important group of shrimp pathogens and their pathogenicity is often attributed to their ability to produce toxins.

The filamentous bacteria such as *Leucothrix mucor, Thiothrix, Flexibacter, etc.,* are often associated with shrimp that are grown in poorly managed farms. These organisms colonise shrimp body surface, gills and appendages in larvae and affect their respiration, feeding, locomotion and moulting. Unlike other bacterial pathogens, these bacteria do not invade shrimp tissues. Mortality of infested shrimp often occurs due to hypoxia. The filamentous bacterial infestation is often associated with other fouling organisms such as ciliate protozoa, and filamentous blue green algae.

Vibrios are ubiquitous aquatic inhabitants and are often reported as causative agents of shrimp disease. They are found on the cuticle, gills and other body surfaces. Vibrios are regarded as secondary pathogens, although some reports on their primary etiology do exist. Attempts to prove Koch's postulates for *Vibrio* spp. that are isolated from outbreaks of vibriosis have seldom been successful. Some studies have suggested that the ability to produce toxins confer their pathogenicity. Example: infectivity and toxicity of *V. harveyi* is mediated by bacteriophage, which is reported to transfer toxin gene. Vibrios have been reported to be associated with the following diseases.

- Luminescent bacterial disease (*V. harveyi*)
- Systemic vibriosis in grow-out ponds
- Seagull syndrome (seagulls and other birds flock the edges of the ponds to feed on dead and moribund shrimp, which die due to vibriosis. Histological sections of lymphoid organ, heart, hepatopancreas, antennal gland, ventral nerve cord, sub-cuticular connective tissue and muscle show multifocal necrosis and septic haemocytic nodules)
- Bolita's syndrome (Spanish: little balls. Attributed to toxigenic *Vibrio* spp. Histological sections of gut and hepatopancreas show sloughed epithelial cells)

### *5.1. Isolation of bacteria*

Demonstration of bacterial agent involved in disease is done by culturing the bacteria from samples of afflicted organs and haemolymph of moribund shrimp from affected ponds. The bacterial pathogens of shrimp are usually *Vibrio* spp. *Pseudomonas* spp. and occasionally *Aeromonas* spp. These bacteria can be easily grown on simple media such as ZoBell's Marine Agar (ZMA) or Thiosulfate citrate bile salts sucrose (TCBS) agar. In place of ZMA, media like tryptose soya agar or nutrient agar supplemented with 1.5-2 % NaCl or the medium prepared in aged seawater can be also used. TCBS, TSA and nutrient agar support the growth of most of the heterotrophic bacteria present in the aquatic environment and on shrimp, while TCBS selectively supports the growth of vibrios only.

*Samples from shrimp:* When a systemic infection is suspected in shrimp of about more than 2 g size, haemolymph sample is drawn either from ventral sinus or directly from the heart using a sterile 26 gauge needle fitted to a tuberculin syringe after disinfection of body surface using 70 % ethanol. The samples of internal organs are taken for bacteriological analysis with the help of sterile bacteriological loop or a sterile forcep. Larval samples are processed after surface disinfection by washing them initially in 25 ppm chlorine containing phosphate buffered saline (PBS) at pH 7.5 for 1 minute, 70 % ethanol for about a minute and then in sterile PBS (pH 7.5) or in sterile seawater for at least three times.

The haemolymph sample, tissue or larval samples are inoculated on to the culture media by streak plate method. The culture plates are incubated at 25-30° C for 18-24 hours and examined for development of bacterial colonies. The colony morphology of bacteria on ZMA, TSA and nutrient agar are recorded. The culture plates are also examined in a dark room for luminescence.

### *5.2. Cultural and morphological characters of bacteria*

For identification of bacteria, some general cultural and morphological characteristics like size, shape, pigmentation, opacity of the bacterial colonies on the solid media; cell shape (rods, cocci, coccobacilli, comma), sporulation, etc., are important and aid in preliminary grouping of bacteria. The morphological characteristics useful for distinguishing bacteria are given in the Table below. Microscopic examination of Gram stained slides of pure culture of bacteria would provide details on shape, arrangement and presence of spores. Size can be measured using ocular micrometer. Separate staining techniques are required for studying flagella and capsule of bacteria.

**Table: Morphological characteristics of bacteria**

| Parameter | Description |
|---|---|
| Shape | Cocci, spherical, oval, short rods, long rods, filamentous, comma, spiral, etc. |
| Size | Length and breadth in µm |
| Arrangement | Single, pairs, chains, in fours (tetrads), in groups, grape like clusters, bundles, irregular |
| Irregular forms | Variation in shape and size, clubs, filaments, branched, etc. |
| Flagella | Polar, monotrichous, amphitrichous, peritrichus, etc. |
| Fimbriae | Polar, peritrichus (EM study) |
| Spores | Spherical, oval, elliptical, sub-terminal, single or multiple |
| Capsule | Present or absent |
| Staining | Reaction to Gram stain |

### *5.3. Identification of bacteria*

Bacteria are identified based on their morphological, physiological, biochemical and serological characteristics. During recent times, specific PCR assays have been also employed for identification of certain bacteria such as *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, etc. Commercial kits for identification of bacteria such as API biolog are also available. These kits comprise miniaturized biochemical tests as dehydrated substrates on plastic strips or in microtitre plates. Pure cultures of bacteria are inoculated and incubated at optimal temperature. Some of the reactions are indicated by colour change and some others have to be added with reagents in order to read the results. The kits are designed for identification of medically important bacteria. Their application in aquaculture is still very limited until suitable database and reactions are evolved for environmental vibrios. Hence, conventional techniques are still very useful for the identification of bacteria isolated from aquaculture environments.

A minimum number of essential physiological and biochemical tests are described under the succeeding paragraphs, which are required to be performed on each of the pure cultures of bacterial isolates from samples of infected shrimp or fish in order to identify

them. The tests included under this section mainly focus on those that are required for identification of species of the genus *Vibrio*, since these are the primary etiologic agents of disease in shrimp and fish. Based on these physiological and biochemical characteristics, the bacterial isolates are identified using the identification schemes (Alsina and Blanch, 1994).

### 5.4. Characterisation of bacteria

#### Gram's Staining

***Principle*:** Staining bacteria by Gram's method is widely used for classification of bacteria into Gram positive and Gram negative bacteria. The bacterial cell walls contain peptidoglycans, which is a thick layer in the Gram-positive bacteria. The pararosaniline dye such as crystal violet treated with iodine mordant remains trapped in the cell wall and hence the cells are not de-stained upon treatment with alcohol.

***Procedure*:**

i. Prepare smears of bacteria on clean glass slide using sterile nichrome loop by mixing with a drop of sterile normal saline.
ii. Fix the smears by air-drying or by gently passing the slide over a Bunsen flame.
iii. Stain the smears with crystal violet solution for one minute.
iv. Wash in tap water for few seconds.
v. Flood the smears with iodine solution for 30 seconds.
vi. Wash in tap water for 15 seconds.
vii. De-colorize with 95 % ethyl alcohol for 30 seconds.
viii. Wash with tap water.
ix. Counter-stain with safranin solution for 10 seconds.
x. Wash in tap water. Blot dry and examine under oil immersion objective of the microscope.

***Interpretation:*** Violet coloured bacteria: Gram positive; Red/pink coloured bacteria: Gram negative. Record size, shape arrangement and other morphological characteristics.

#### Motility test (Hanging drop method)

i. Place a very small drop of log phase broth culture of bacteria with the help of sterile inoculating loop (2 mm dia) at the centre of a cover glass.
ii. Place small drops of water on the corners of the cover glass.
iii. Invert the cover glass over the cavity of the slide, so that the drop of culture is hanging at the centre of the cavity slide.
iv. Observe the hanging drop of bacterial culture under microscope for mortility of bacteria.
v. Darting or zig-zag motility indicates that the bacteria may have polar flagellation, while slow motility or vibratory motility indicates peritrichous flagellation. These have to be distinguished from Brownian movement.

**Oxidase test**

***Principle:*** Some bacteria possess cytochrome oxidase or indophenol oxidase, which catalyses transport of electrons from donor compounds to oxygen. In this test, the N,N,N',N' tetramethyl p-phenelene diamine dihydrochloride, a colourless dye serves as an artificial electron acceptor. The oxidase enzyme produced by bacteria oxidises the dye producing coloured indophenol blue.

***Procedure*:**

i. Place a strip of whatman No.1 filter paper in a petri dish.

ii. Add 2-3 drops of freshly prepared 1 % solution of N,N,N',N'- tetramethyl paraphenylene diamine dihydrochloride.

iii. Smear the test colony of bacteria on the filter paper using a sterile capillary or platinum loop.

***Interpretation:*** Positive reaction is indicated by development of a deep purple colour of the smear within 10 seconds.

**Catalase test**

***Principle*:** Bacteria possess an enzyme called catalase which catalyses breakdown of toxic hydrogen peroxide ($H_20_2$) formed during the cell's metabolism into water and oxygen. When a solution of $H_2O_2$ is added to bacterial cell suspension, the catalase is activated, resulting in the release of $O_2$, which is observed as effervescence.

***Procedure:***

i. Make a drop of heavy suspension of test culture of bacteria on a slide.

ii. Place a drop of 10 % hydrogen peroxide solution over the bacterial suspension.

iii. Observe for small air bubbles.

***Interpretation:*** Production of gas bubbles (effervescence) indicates a positive reaction.

**Carbohydrate fermentation test**

***Principle:*** When the bacteria are grown in basal media containing specific carbohydrates such as glucose, sucrose, lactose, mannitol, etc., in the presence of a pH indicator, manifest into colour change depending on the metabolic pathway used by the bacteria.

***Procedure:***

i. Inoculate the bacterial isolate in duplicate into phenol red broth base incorporated with sugars such as glucose, lactose, mannitol, etc. in sugar fermentation tubes.

ii. Overlay one tube with sterile mineral oil (*e.g*. liquid paraffin) about 1-2 cm. Incubate the tubes at 28° C.

iii. Observe the tubes for colour change at 24, 48 and 72 hours intervals.

***Interpretation:*** Acid production in open tube indicates oxidative metabolism and acid production in the tube overlaid with mineral oil indicates fermentative metabolism of the bacteria.

**Decarboxylation of aminoacids**

***Principle*:** Some bacteria are able to produce enzymes that attack carboxyl group of amino acids. The reaction is anaerobic. Bacteria are inoculated into tubes containing the amino acid along with a control tube, which contains only the basal medium without the amino acid. The tubes are made anaerobic by overlaying with sterile mineral oil over the medium. If the test organism does not produce decarboxylase, both the control and test tubes turn yellow due to fermentation of small amount of glucose present in the medium yielding acidic products, lowering the pH of the medium. If the amino acid is decarboxylated, the tubes revert to original purple colour, because of the alkaline amines produced during the reaction, which increase the pH of the medium.

***Procedure:***

i. Inoculate the tubes of Moeller's decarboxylase medium containing appropriate amino acid (lysine/arginine/ornithine) along with a control tube without amino acid with bacterial isolates.

ii. Overlay the tube containing amino acid with 2-3 cm mineral oil (liquid paraffin).

iii. Incubate the tubes at 28° C and observe daily for four days for change of colour.

***Interpretation:*** Purple (original colour of the medium, alkaline reaction) indicates decarboxylation of lysine and ornithine and positive reaction for arginine dihydrolase. Yellow indicates fermentation of glucose only and negative reaction for decarboxylase and dihydrolase.

**O-Nitrophenyl-β-D-galactopyranoside (ONPG) hydrolysis test**

***Principle*:** The test demonstrates the ability of bacteria to ferment lactose. Two enzymes are involved in this activity. The permease permits the lactose molecule into the cell, while the β-galactosidase hydrolyses lactose to galactose and glucose. Some bacteria lack the ability to produce permease and possess the enzyme β-galactosidase. The ONPG, a compound similar to lactose molecule is hydrolysed by the enzyme β-galactosidase into galactose and o-nitro phenyl, which is a yellow compound.

***Procedure*:** Inoculate heavily a tube containing ONPG broth with the bacterial culture. Incubate at 28° C for 1-2 hour. Examine the colour change of the broth from colourless to yellow.

***Interpretation:*** Development of yellow colour indicates positive activity for β-galactosidase activity (fermentation of lactose).

**Nitrate reduction**

***Principle:*** Bacteria can assimilate inorganic nitrate into their proteins by virtue of one of the enzymes in a complex process called nitrate reductase, which converts nitrate ($NO_3$) to nitrite ($NO_2$). $NO_2$ is detected by an inorganic assay using α-naphthylamine and sulfanilic acid.

***Procedure:***

i. Inoculate test culture of bacteria to tubes containing about 2 ml of nutrient broth supplemented with 0.1 % $KNO_3$.
ii. Incubate at 28° C for 24 h.
iii. Add 1 ml each of alpha napathylamine solution and sulfanilic acid solution.

***Interpretation:*** Positive reaction (conversion of $NO_3$ to $NO_2$) is indicated by development of pink colour.

*Note*: When there is no development of pink colouration, add a pinch of zinc dust. If pink colouration develops, it suggests that $NO_3$ is left without reduction to $NO_2$.

**Indole test**

***Principle:*** Indole is produced upon degradation of tryptophan by some bacteria. Production of indole is detected by formation of pink coloured compound when it reacts with an aldehyde such as p-dimethyl amino benzaldehyde.

***Procedure:***

i. Grow bacteria in 1 % peptone water broth for 24 h at 28° C.
ii. Observe for turbidity, which is indicative of growth.
iii. Add 0.5 ml of Kovac's reagent to the broth culture of bacteria and shake gently.
iv. Observe for development of pink colour.

***Interpretation:*** Development of pink colour indicates a positive reaction.

**Voges Proskauer's test**

***Principle*:** The test detects acetoin or acetyl methyl carbinol, an intermediate product in the formation of butylene glycol during the metabolism of glucose. Acetoin is oxidised to diacetyl in the presence of oxygen by potassium or sodium hydroxide, which is a red coloured complex. Sensitivity of the test is further improved by addition of α-naphthol prior to addition of KOH.

***Procedure*:**

i. Grow pure culture of the bacteria in 5 ml of MR-VP broth at 28° C for 48 hours.
ii. Transfer about 2.5 ml of culture to another tube.
iii. Add 0.3 ml of alcoholic α-naphthol and 0.1 ml of 40 % KOH solution, gently agitate the tube and wait for one hour.
iv. Observe for formation of red colour.

***Interpretation*:** Development of red/crimson colour indicates that bacteria produce acetyl methyl carbinol.

**Salt tolerance**

***Principle*:** Various species of *Vibrio* and related bacteria can be differentiated based on their ability to grow in the presence of different levels of sodium chloride.

***Procedure:*** Inoculate bacterial culture into nutrient broth tubes containing 0, 3, 6, 8 and 10 % NaCl. Incubate at 37° C overnight and observe for growth, which is indicated by turbidity of the broth, compared to un-inoculated control.

**Sensitivity to O/129**

***Principle:*** *Vibrio* species are sensitive to 150 µg of O/129 (2,4 di amino, 6-7 di isopropyl pteridine), while the other related genera like *Pseudomonas, Aeromonas, Plesiomonas, Alkaligenes,* etc., are resistant. The test is done by using O/129 impregnated discs employing conventional disc diffusion method.

***Preparation of O/129 discs:*** Prepare 7500 and 500 µg/ml (7.5 mg and 0.5 mg respectively) in sterile glass double distilled water. Spot 20 µl of these stock solutions onto sterile antibiotic discs to obtain discs containing 150 or 10 µg O/129. Dry the discs in a desiccator under aseptic conditions at room temperature. Store at 4° C till use.

***Procedure*:** Test sensitivity of the bacterial isolates as the protocol given for antibiotic sensitivity testing.

***Interpretation:*** Zone of inhibition of growth around the O/129 impregnated discs indicates susceptibility of bacterial isolate to O/129.

**Antibiotic/drug sensitivity testing**

The antibiotic/drug sensitivity testing method employed is Kirby-Bauer's disc diffusion technique. Commercially available antibiotic discs are used for this purpose. The culture medium used for antibiotic sensitivity testing is Mueller-Hinton agar supplemented with 1 % sodium chloride.

***Preparation of the inoculum:*** Inoculate pure culture of bacteria into 5 ml ZoBell's marine broth tubes with the help of sterile inoculation loop. Incubate for 2 to 8 hours at 28° C till moderate growth is obtained.

***Note:*** Obtain turbidity of broth culture (by diluting the culture using sterile sea water or sterile phosphate buffered saline, pH 7.4) equivalent to 0.5 ml of 1.175 % $BaCl_2.2\ H_2O$ solution added to 99.5 ml of 0.36 N sulphuric acid.

***Inoculation:*** Dip a sterile swab into the inoculum and squeeze off the excess fluid by pressing the swab against the inside wall of the tube. Streak the entire agar plate thoroughly on the surface.

***Application of antibiotic discs:*** Apply discs onto the plates aseptically using sterile forceps. Press the discs firmly on the agar to enable smooth diffusion of antibiotic. Place the antibiotic discs at least 20 mm apart. Incubate the plates at 30° C.

Examine the plates after 24 hours. Measure the zone of inhibition and record. See the zone interpretative chart given by the supplier of antibiotic discs and record as sensitive or resistant.

### 5.5. Estimation of total viable bacterial count in water sample

Estimation of total viable count (TVC) is used to determine the density of living bacteria in a sample. Serial dilution of the water sample followed by spread plate or pour plate method is employed to achieve this objective. The sample is diluted serially in sterile normal saline solution. The serial dilution helps to reduce the number of bacteria in the medium to manageable limit as the plates showing 30-300 colonies are considered countable.

When a diluted sample is plated, the number of colonies produced can be used to calculate the original cell density in the sample using the formula as follows:

$$\text{Bacterial count (TVC)} = \frac{\text{No. of colonies counted x Dilution factor}}{\text{ml of sample plated}}$$

Since it is virtually impossible to know if the colonies produced on plates originated with a single cell or a cluster of cells, the term colony forming unit (cfu)/ml is used instead of bacteria/ml.

Example:

Say the number of colonies counted on the plate = 55

Volume of diluted sample plated = 0.1 ml

Dilution factor in the tube from which the sample was taken for plating = $10^{-5}$

Hence total no. of bacteria in the sample = $55 \times 10^5 = \frac{5500000}{0.1}$

$= 5.5 \times 10^7$ cfu/ml

**Green colonies and yellow colonies on TCBS**

During recent years, a number of shrimp farmers have begun to monitor bacterial counts in shrimp culture ponds. As a sequel, a myth has been developed among shrimp farmers that ponds having high counts of green colonies on TCBS are good and those growing yellow colonies are bad. The TCBS medium is designed to selectively culture *Vibrio* species. The medium contains a di-saccharide sucrose and bromothymol blue as pH indicator. Some species such as *V. alginolyticus, V. fluvialis, V. anguillarum* and *V. cholerae* ferment sucrose resulting in the formation of acid, giving the colonies yellow appearance because of change in the pH indicated by the bromothymol blue. *V. campbelli, V. parahaemolyticus, V. vilnificus* do not ferment sucrose and hence produce green colonies on TCBS. The presumption of farmers that yellow colonies are bad is because of the increased reports of vibriosis associated with *V. alginolyticus* and *V. anguillarum*. However, other species can also cause vibriosis, since, they are opportunistic pathogens.

## Culture Media, Reagents and Stains

### Culture Media

i. ZoBell's marine agar
*ii.* *Aeromonas* selective medium
*iii.* *Pseudomonas* selective medium
iv. ZoBell's marine broth
v. Nutrient broth
vi. Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar
vii. Mycological agar
viii. Sabauraud's dextrose agar
ix. Marine Oxidation Fermentation Medium (MOF)
x. Decarboxylase base (for testing decarboxylation of amino acids)
xi. MR-VP medium
xii. Phenol red broth base
xiii. Amino acids: lysine, arginine and ornithine; NaCl, NaOH, and other required chemicals. These items can be obtained as dehydrated powders from commercial sources.

***Medium for isolation of bioluminescent bacteria***

| | |
|---|---|
| Peptone: | 5.0 g |
| Yeast extract: | 3.0 g |
| Glycerol: | 3.0 ml |
| Agar: | 15.0 g |
| Distilled water: | 250 ml |
| Aged seawater: | 750 ml |

Dissolve the ingredients and adjust pH to 7.8; autoclave at 15 lb for 15 minutes, cool to 48° C, pour plates.

***Peptone water***

| | |
|---|---|
| Peptone: | 1.0 g |
| *NaCl: | 0.5 g |
| Distilled water: | 100 ml |

**(*Increase the NaCl concentration from 1.0 to 1.5 % when working with bacterial isolates of brackishwater or marine environment)**

***ONPG Broth***

1. ONPG solution: 0.6 g

   *Sodium phosphate buffer: 100 ml.

(*Dissolve 13.8 g sodium phosphate ($Na_2HPO_4$) in 50 ml of warm distilled water in a volumetric flask. Add distilled water to make up to about 80 ml. Adjust the pH to 7.0 with 5N NaOH. Make the volume up to 100 ml. Filter sterilize the solution. Store in refrigerator in a dark brown bottle)

2. Add 25 ml of ONPG solution to 75 ml peptone water. Dispense 0.5 ml volumes in sterile tubes.

**Reagents**

1. Reagents for nitrate reduction test
   Solution A: alpha-napathylamine: 1 g
   Distilled water: 20 ml
   Dissolve, filter and add 180 ml of 5 N acetic acid
   Solution B: Sulfalinic acid: 0.5 g
   5 N acetic acid: 150 ml.
2. Kovac's reagent for Indole test
   n-amyl or n-butyl alcohol: 150 ml
   Para dimethyl amino benzaldehyde: 10 g
   Conc. HCl: 50 ml
   Dissolve the aldehyde in alcohol and slowly add acid.
3. Reagent for Voges Proskauer's test
   A: 5 % alpha-napthol in absolute alcohol
   B: 40 % KOH or NaOH

**Stains**

For Gram's staining

1. Solution A: Crystal violet: 2 g
   Ethyl alcohol (95 %): 20 ml
   Solution B : Ammonium oxalate: 0.8 g
   Distilled water: 90 ml
   Mix both solutions and filter
2. Gram's iodine
   Iodine: 1 g
   Potassium iodide: 2 g
   Distilled water: 300 ml
3. Safranin solution
   Safranin (2.5 % solution in 95 % ethyl alcohol): 10 ml
   Distilled water: 100 ml

## 6. Immunodiagnostic techniques

Immunological techniques have contributed to a great extent in the diagnosis of infectious diseases in human and veterinary medicine. However, these techniques are yet to make an impact in disease diagnosis in aquaculture. This is because of the fact that the immune system of fish and crustacea is primitive and different from vertebrates. The immune response in animals (vertebrates) is classified into two types: a) humoral, and b) cell mediated. The humoral immune response is the one involving development of antibodies. The tests targeted against these antibodies in the humoral immune response are used for developing immunodiagnostic tests.

The basic principle behind most of the immunological tests is that a specific antigen will combine with its specific antibody to form an antigen - antibody complex, which is usually insoluble and may be seen with the naked eye. The immunochemical tests range from simple manual methods to fully automated systems with sophisticated integrated detection. Enzymes are also widely used as labels in immunoassays for colourimetric, fluorimetric, and chemiluminescent detection.

The antibodies required for the tests are produced conventionally in rabbits or guinea pigs or mice by inducing antibody production upon injection of antigen (pathogenic bacteria or virus in pure form). The serum from sensitized animal is harvested, which will contain the specific antibodies. The antibodies produced by these conventional methods would possess a heterogeneous population of immunoglobulins (antibodies) from several clones of B cells. The tests developed based on such polyclonal antibodies are less sensitive. However, monoclonal antibody technology is used to produce homogenous antibodies through immortalizing single clones of antibody producing cells from immunized animals by fusing with myeloma cells. This development has facilitated enhancement of sensitivity and specificity of immunochemical techniques profoundly.

Today, a number of immunochemical tests are available, which can be applied to aquaculture.

### 6.1. Agglutination tests

When the antigen is particulate, the reaction of an antibody with the antigen can be detected by agglutination (clumping) of the antigen. When the antigen is an erythrocyte, the term hemagglutination is used. All antibodies can theoretically agglutinate particulate antigens. However, among all categories of antibody molecules, IgM has exceptionally good agglutination property due to its high valency. The antibody is mixed with the particulate antigen and a positive test is indicated by the agglutination of the particulate antigen. Agglutination tests can also be used for quantification of the level of antibodies to particulate antigens. Serial dilutions of a sample serum to be tested for antibody are added to a fixed number of bacteria or other such particulate antigen, and the maximum dilution of the antiserum, which gives agglutination is determined. The maximum dilution that gives visible agglutination is called the antibody titer. Although the test is easy to perform, it is only semi-quantitative.

Agglutination tests are applied for 1) determination of blood types or antibodies to blood group antigens; 2) to assess bacterial infection, *e.g.*, a rise in titer to a particular bacterium indicates an infection with that bacterial type. A fourfold rise in titer is generally taken as a significant rise in antibody titer.

### 6.2. Precipitation tests

***Radial immuno-diffusion:*** In radial immuno-diffusion, antibody is incorporated into the agar gel as it is poured and different dilutions of the antigen are placed in wells punched into the agar. As the antigen diffuses into the gel, it reacts with the antibody and when the equivalence point is reached a ring of precipitation is formed.

Diameter of the ring is proportional to the log of the concentration of antigen since the amount of antibody is constant. At maximum precipitation stage, the area circumscried by the immunoprecipitation ring is positively linearly correlated with the amount of antigen applied to the well and negatively linearly correlated with the concentration of Ab in the gel. Thus, by running different concentrations of a standard antigen one can generate a standard curve for quantification of an antigen in an unknown sample. If more than one ring appears in the test, more than one antigen/antibody reaction has occurred. This could be due to a mixture of antigens or antibodies. This test is commonly used in the clinical laboratories for determination of immunoglobulin levels in blood samples.

***Immunoelectrophoresis:*** In immunoelectrophoresis, a complex mixture of antigens is placed in a well punched out of an agar gel and the antigens are electrophoresed so that the antigens are separated according to their charge. After electrophoresis, a trough is cut in the gel and antibodies are added. With the diffusing of the antibodies into the agar, precipitin lines are produced as the antigen antibody reactions occur. Immuno-electrophoresis is used for the qualitative analysis of complex mixtures of antigens. This test is commonly used for the analysis of components in serum.

***Countercurrent electrophoresis:*** In this test the antigen and antibody are placed in wells punched out of an agar gel and the antigen and antibody are electrophoresed into each other where they form a precipitation line. This test only works if conditions can be found where the antigen and antibody have opposite charges.

### 6.3. Immunofluorescence

Immunofluorescence is a technique whereby an antibody labeled with a fluorescent molecule (fluorescein or rhodamine) is used to detect the presence of an antigen in or on a cell or tissue by the fluorescence emitted by the bound antibody.

a) Direct immunofluorescence– In direct immunofluorescence, the antibody specific to the antigen is directly tagged with the fluorochrome.

b) Indirect immunofluorescence– In indirect immunofluorescence, the antibody specific for the antigen is unlabeled and a second anti-immunoglobulin antibody directed toward the first antibody is tagged with the fluorochrome. Indirect

fluorescence is more sensitive than direct immunofluorescence since there is amplification of the signal.

***Radioimmunoassay (RIA):*** Radioimmunoassays (RIA) are based on the measurement of radioactivity associated with immune complexes. In any particular test, the label may be on either the antigen or the antibody.

### 6.4. Enzyme-linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assays (ELISA) are those that are based on the measurement of an enzymatic reaction associated with immune complexes. The enzyme may be linked to either the antigen or the antibody. The test can be used for qualitative and quantative detection of either antigen or antibodies. The test is usually performed in microtiter plates made of polystyrene. An ELISA utilizes the binding of anti-mouse IgG antibody conjugated to an enzyme and the subsequent production of a colored product to detect the presence of an antibody, which recognizes the antigen. The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen (as in hybridoma screening). An ELISA is a five-step procedure. The first step involves coating the microtiter plate wells with antigen. The second step includes blocking of all unbound sites, to prevent false positive results. The third step is the addition of antibody to the wells. The fourth step is the addition of anti-mouse IgG conjugated to an enzyme. The fifth step involves the reaction of a substrate with the enzyme to produce a colored product, thus indicating a positive reaction. Enzymes such as alkaline phosphatase conjugated to anti-mouse IgG are most commonly used; and it is used with a substrate solvent, diethanolamine. Horseradish peroxidase is also often used. It is about 10 times more sensitive than alkaline phosphatase and is used with 'safe' color development solution. Hence, it can be used to detect very low levels of antibody. Because of its high sensitivity, the color produced is more variable, perhaps due to particles introduced from the air, inadequate rinsing or temperature variability.

### 6.5. Immunodiagnostic tests for diseases of shrimp

| Test | Pathogen | Reference |
|---|---|---|
| ELISA | WSSV | Nadala *et al.*, 2000 |
| Dot immunoassay | WSSV | Nadala *et al.*, 2000; Poulos *et al.*, 2001 |
| | TSV | Poulos *et al.*, 1999 |
| | YHV | Sithigorngul *et al.*, 2002 |
| Immuno-electron microscopy | WSSV | Zhan *et al.*, 1999 |

## 7. Cell culture

The role of cell culture in disease diagnosis is limited to the extent of isolation of the virus *in vitro*. The samples from infected fish and shrimp are prepared by homogenizing the target tissues such as brain (nervous tissue) and passed through 0.22 µmm membrane filters.

The filtrate is inoculated to monolayers of established cell lines grown in tissue culture media like Lebovitz L-15 medium or Eagle's minimum essential medium (MEM) and incubated at optimal temperature. The cell lines are examined for development of cytopathic effects (CPE) daily for a period of about two weeks. The CPE caused depends on the type of virus. Cell rounding, granulation, vacuolation in cytoplasm, partial or complete disintegration of cells of monolayer are some of the cytopathic effects caused by viruses. Upon observation of CPE, diagnosis of disease would be narrowed down to viral infection, the identity of which can be confirmed by electron microscopy or immunological or molecular methods.

A number of cell lines derived from fish are available for studying viruses affecting fish. GF-1 cell line derived from grouper has been reported to be susceptible to grouper nervous necrosis virus and SSN-1 cell line can support the growth of piscine nodaviruses. The viruses associated with viral haemorrhagic necrosis can be isolated in BF-2, RTG-2 or EPC cell lines. RTG-2, GF, FHM and BB cell lines are available from the National Centre for Cell Science, Pune, India.

However, the marine invertebrate cell culture has become a challenge to the scientific community. So far, no single established cell line is available either for disease diagnostic purpose or for propagation of crustacean viruses. Attempts have been made for the establishment of cell lines derived from various tissues such as haematopoietic tissue, lymphoid organ, gonads, nerve cells and haemocytes. Most of these attempts could establish cell cultures of short duration only.

## 8 Bioassay

The term bioassay is usually employed for toxicity testing with different organisms. A bioassay uses a living organism, usually a plant or bacteria as a test agent for the presence or concentration of a chemical compound or a disease. The idea is to choose a test agent that is very sensitive to the condition being tested. In the present context, bioassay is applicable for the presumptive diagnosis of diseases of viral aetiology. After other aetiologies like bacteria, fungi and parasites are ruled out, bioassay can be conducted to demonstrate that the aetiology of disease is a viral agent. In order to do this, specific tissues of live shrimp/ fish samples are homogenised and filtered through 0.22 µmm pore size filters, which removes bacteria and larger particles, while retaining the viruses. Fish/shrimp are exposed to the filtrate through injection or feeding and observed for development of disease.

## 9 Electron Microscopy (EM)

### *9. 1 Transmission EM*

#### *9.1. 1 Specimen processing*

##### *9.1.1.2 Sampling*

Usually soon after the death of the organism, post mortem changes will take place making the tissue unsuitable for ultra structure studies. Hence, for electron microscopy, live animals are preferred. The animals are sacrificed, desired tissues/samples dissected out

and immediately placed in cold fixative. The desired size of the tissue to achieve proper fixation is about 1 mm. Small animals and larvae of less than 2 mm size are fixed in ice-cold fixative as a whole in live condition. The sample vial should be labeled properly.

### *9.1.1.3. Fixatives*

Fixatives help to preserve the structures in the living cells and prevent the changes induced by autolysis. There is no single ideal fixative and so a combination of fixatives is preferred depending on the type and nature of the tissues. In electron microscopy, 2-4 % Gluteraldehyde is used as the primary fixative, which is excellent in fixing nucleic acids, nuclear proteins and carbohydrates but not lipids. Poor contrast and slow penetration are the limiting factors of gluteraldehyde fixative. Osmium tetroxide is used for secondary fixation. It acts as both fixative as well as stain, fixes nucleic acids, carbohydrates and lipids and provides contrast and fast penetration. The combination of gluteraldehyde and osmium tetroxide as primary and secondary fixatives, gives the desired results in contrast and resolution.

Fixatives are prepared in a suitable buffer for two reasons, to maintain the pH (7.2 to 7.4) and to maintain the osmolality, in order to minimize the swelling or shrinkage of the tissues, which may otherwise lead to artifacts. The most commonly used buffer is sodium cacodylate buffer.

### *9.1.1.4. Primary fixation*

Tissues are fixed in 2 to 4 % gluteraldehyde in 0.1 M cacodylate buffer (in the case of marine species, 5 to 3 % NaCl or sucrose can be added to the fixative). For proper penetration of the fixative, the tissues should not exceed 1 mm in size. Fixation is carried out for 4-6 hours (varies depending on the nature of the tissues), at 4° C. After fixation, the fixative is drained and tissues washed thrice (15 minutes each) with buffer. In case of larger tissues, further trimming is done if required and washed with fresh buffer.

***9.1.1.5. Secondary fixation or post fixation:*** For secondary or post fixation, the washed tissues are transferred to 1 % osmium tetroxide ($OsO_4$) in 0.1M cacodylate buffer, kept for 1-2 hrs at 4° C (above 4° C, $OsO_4$ disintegrates). $OsO_4$ treatment turns the tissues black. $OsO_4$ is drained off and tissues washed two to three times with buffer, for 15 minutes each, or until free of a black precipitate formed from excess $OsO_4$. Samples can be stored in buffer under refrigeration until further processing is desired (Since $OsO_4$ is highly toxic, care must be taken while handling). Always use gloves and carry out all operations under a hood).

***9.1.1.6. Dehydration:*** Dehydration is done through graded alcohol or acetone series to remove the water from the tissues.

Dehydration can be done as follows

| | |
|---|---|
| 30 % Acetone (analar) | two changes, 15 minutes each at 4° C |
| 50 % Acetone (analar) | two changes, 15 minutes each at 4° C |

| | |
|---|---|
| 70 % Acetone (analar) | two changes, 15 minutes each at 4° C, (tissues can be stored in 70% acetone indefinitely, until further processing) |
| 80 % Acetone (analar) | two changes, 15 minutes each at 4° C |
| 90 % Acetone (analar) | two changes, 15 minutes each at 4° C |
| 95 % Acetone (analar) | two changes, 15 minutes each, 4° C |
| 100 % Acetone (analar) | two changes, 15 minutes each, 4° C |
| 100 % Acetone (analar) | two changes, 30 minutes each at room temperature |
| Propylene oxide | two changes of 15 minutes each at room temperature |

***9.1.1.7 Infiltration and embedding:*** Fixed and dehydrated tissues are infiltrated with liquid plastic resins and then cast into blocks. The purpose of embedding is to allow future ultrathin sectioning of the material. Commercially available plastic resins like Epon or Spurr are used for embedding. The media are mixed as per the instructions under a fume hood. Prepare fresh media 2-3 hours prior to use, as it will absorb the water vapors from the surroundings and the components will begin to polymerize. Mixture of embedding medium (Spurr's medium) and acetone is prepared in various grades (mixture A– medium and acetone in the ratio 1: 3, mixture B– medium and acetone ratio 2: 2 and mixture C– medium and acetone in the ratio 3: 1) and the tissue is kept in each for 1-2 hours each or as specified (period varies with the medium used) for infiltration. For embedding, the medium is prepared as instructed, poured into readymade moulds made of plastic or silicon rubber and infiltrated tissues transferred to it, taking care not to trap any air bubble. The moulds are then kept in an incubator at 70° C for 12 to 24 hours.

Each tissue with reference to the experimental objective requires an evaluation of the methods, subjected to a careful examination of pertinent literature. There is no schedule that will work for all tissues and conditions.

***9.1.1.8 Trimming:*** The resin blocks are trimmed to remove the unwanted areas using a glass knife fitted to an Ultramicrotome.

***9.1.1.9 Sectioning and staining:*** To achieve high resolution for electron microscopy, the sections should be very thin (60 nm) and prepared using an ultramicrotome. Standard procedures are followed for obtaining semi-thin and ultrathin sections for light and electron microscopy respectively. Semi-thin sections are first taken, stained and observed under a light microscope for determining the area for ultrathin sectioning. The blocks are again trimmed and ultra-thin sections taken. These sections are floated on distilled water, stretched to remove the wrinkles and collected over the matty/dull surface of the copper or nickel grid.

***9.1.1.10 Staining:*** Double staining with uranyl acetate and lead citrate is employed for routine electron microscopy studies. The sections are first stained with uranyl acetate.

A drop of uranyl acetate (saturated solution in 50 % ethanol) is taken on a clean glass slide and the grid with the section side down is kept on to the stain drop and is covered with any opaque object to ensure darkness to carry out the staining effectively. After 10-15 minutes, the grid is taken out and washed 3-4 times in double distilled water (ensuring that the sections are not washed away) and dried with a filter paper. The grids are then stained with lead citrate for 1-4 minutes, washed well and dried. In the case of a particulate specimen, it is taken on formvar-coated grids, subjected to negative staining using 1-3 % phosphotungstic acid and dried.

***9.1.1.11 Observation and photography:*** The grid carrying the stained section is loaded into the electron microscope, the image observed and recorded on photographic plates/ film. In order to study and interpret EM results, a thorough knowledge about the ultrastructure of the normal cells and the pathogen is essential.

## 9.2 Scanning Electron Microscopy (SEM)

The scanning electron microscope, like the Transmission Electron Microscopy (TEM) consists of an electron optical column, a vaccum system and works under the same principle as that of the TEM. The electron gun produces an extremely fine beam of electrons, which are focused on a fine spot less than 4 nm on the specimen and scanned in a rectangular raster over the specimen. The secondary electrons produced by the interaction of the electron beam with the specimen surface as well as the backscattered primary electrons (depending upon the topography) are detected by a suitable sensor/ detector. The signals from the detector are electronically amplified to modulate the brightness of a cathode ray tube (CRT) so as to produce an image, which can be recorded photographically.

***9.2.1 Specimen preparation for SEM:*** The specimen is first fixed with gluteraldehyde as in the case of TEM and washed well in buffer. Post fixation with $OsO_4$ is optional. The specimen is subjected to dehydration using ascending grades of acetone as in TEM processing. The dehydrated specimen for SEM has to be dried without causing any shrinkage. Except in the case of fine particulate specimen, critical point drying or freeze drying is usually preferred for drying SEM samples. The dried specimen is then coated with a thin conductive metal film (Gold, Palladium, etc.) using an ion coater to prevent charging artifacts and to stabilize the specimen mechanically.

Presently, variable pressure SEMs are available which can operate without high vacuum, thus avoiding time-consuming specimen preparation techniques as well as reduce specimen damage caused during coating**.**

**9.3 Benefits:** The most important among the benefits offered by the electron microscope is undoubtedly the very high resolution (as low as 0.1 nm) and magnification (up to 1000,000X) in TEM and 0.4 nm resolution with a magnification of 800,000X in SEM. Since timely and accurate diagnosis forms the first step in the health management of farmed fin and shellfishes, the right diagnosis defines the very success of disease control. Factors like high cost of operation and infrastructure, need for skilled technical personnel,

laborious and time-consuming procedures, thorough knowledge needed for interpretation, etc., restricts the use of electron microscopy as a routine diagnostic tool. However, as a confirmatory diagnostic method for many of the existing and emerging diseases, especially of viral origin, electron microscopy still remains an indispensable tool in the field of disease investigation and control**.**

## 10. Nucleic acid based diagnostics

Researchers' quest for a novel diagnostic method with high specificity, sensitivity and speed finally found an answer in the emerging molecular biology and recombinant DNA technology. The very basic of the nucleic acid based diagnosis rests in the unique genetic make up or the genetic fingerprint of each organism or pathogen in the form of DNA or RNA. Information generated on the DNA sequence, can be directly used for the development of DNA/RNA based diagnosis.

### *10.1 Nucleic acid probes*

Gene probes consists of single stranded DNA or RNA with a chosen sequence to base pair with complimentary strand under appropriate conditions of temperature, salt and pH, tagged with a proper signal producing substance such as enzymes, fluorescences, antibodies, chemiluminescence or radio isotopes. The probe will bind to its corresponding sequence in the target. The binding of probe and target molecule can result in the formation of a probe-target complex. This complex can be located or identified (diagnosed) by the signals produced by the different types of probe labels.

Radioactive labels such as 32P and 35S, used to be the most common ones (detection by autoradiography or Geiger-Muller counters). However, the short half-life and hazardous nature of the radiochemicals made them less popular. This has stimulated the development of alternative labeling methods based on non-radioactive chemicals, capable of producing different signals. These are safer than the radiolabels and do not require special facilities such as radioactive laboratory. The examples of non-radioactive labels are, colour change (enzymes are attached to the probe, which react to a substrate giving a colour change), antibodies (antigenic group is coupled to the probe and its presence detected using antibodies), fluorescence (chemicals attached to the probe fluoresce under UV light) and chemiluminescence (chemiluminescent chemicals attached to the probe are detected by their light emission). These labels are incorporated into the probe by different methods such as nick translation, primer extension and end-labeling.

Common size range for most probes is between 14 and 40 bases of nucleotides. Long probes may be 100's of bases in length. The probes can be DNA or RNA, but DNA probes are common. The **dot-blot hybridization** and ***in situ*-hybridization are examples of gene probes**, which are increasingly being used in the aquaculture disease diagnosis. Gene probes allow identifying the molecule of interest (pathogen) from the complex mixture of cellular components of both the host and pathogen.

**Dot Blot Hybridization:** Dot blots helps to detect the unfractionated DNA or RNA molecules fixed on a membrane. In **Dot Blot Hybridization,** the gene probe and target (macerated tissue or blood sample) are attached to a neutral solid support (the membrane).

Most commonly used membranes are nitrocellulose and nylon membranes. These two membranes are interchangeable and the choice between them is only personal preference. For dot blots, firstly, samples are boiled to separate the suspected pathogenic DNA strands and then baked onto the membrane. The non-radiocative labeled gene probe is boiled (to separate the strands) and added to the membrane. If the target pathogen DNA is present in the sample, the probe will hybridize (bind) to it. Other unbound probe components are removed by washing. The labeled enzymatic probe will react with the pathogen in the samples, producing a dark dot indicating the infection.

***In situ* hybridization:** *In situ* hybridization enables direct detection of pathogenic nucleic acid in infected specimens. Here, formalin fixed paraffin sections of the target tissue on a microscopic slide is treated with nucleic acid tagged with a fluorescent or enzymatic label, or the probe. The wax is first removed to give access for the probe to the nucleic acid of the pathogen. The slide with tissue and the probe is then heated to separate the DNA strand. If the tissue is infected, the DIG labeled probe specifically binds to the target DNA of the pathogen. The binding of the probe with the pathogenic DNA produces a colour change/precipitation, which can be viewed through a microscope, enabling an apt diagnosis. This technique allows the profile and the distribution of the pathogen (as nucleic acid) in morphologically preserved chromosomes, cells or tissue itself to be seen, which is not possible with other diagnostics, where the tissue structure is destroyed in the process of sample preparation. The *in situ* hybridization technique is widely used as a research tool than a diagnostic tool, as the method is time consuming and laborious.

### *10.2 Polymerase chain reaction (PCR)*

Since the development of PCR by Mullis and co-workers in mid-eighties, this simple *in vitro*-DNA synthesizing technique has opened up new vistas in disease diagnosis and health management. In principle, it is possible to start from a single cell, a single virus or microbe (the genomic content per cell varies from .01pg in prokarytes to 0.3 -10 pg in higher plants and animals) and get enough DNA to analyze (diagnose) by PCR amplification. Major components in the PCR are: Primers, Target DNA, Thermostable DNA polymerase, Magnesium, d NTPs (bases)

***10.2.1 Oligo nucleotide primers*:** Primers are custom made (by a DNA synthesizer) short strands (~17bp- 40bp) of single stranded DNA. They have to be designed as complementary sequences to 'prime' the known sequence of the pathogen of interest. PCR needs two oligonucleotide primers, which bind the DNA sequence that is to be amplified. One primer is complementary to the DNA strand in the forward direction at the beginning of the target sequence, while the second primer is complimentary to the DNA target sequence on the reverse direction at the end of the target sequence. The 3' end of the primers serves as the starting point of DNA amplification. Primer concentration need to put in a PCR has to be standradised with each PCR. High primer-template ratio can results in non-specific amplification and primer di-mer formation.

***Target DNA:*** The target or template DNA is the nucleic acid extracted from the biological specimen of interest (infected tissue, pathogen). Successful amplification of the DNA of

interest depends on the amount and quality of the DNA template used in PCR. Tissue samples (gill or pleopods) for PCR analysis can be fixed in 70-90 % ethanol or maintained in frozen condition (-20$^0$ C). Samples intended to be stored in ethanol need to be given two fluid changes before storage or transportation. The sample ethanol ratio should be 1:10.

***10.2.3 Thermostable DNA polymerase:*** The discovery of thermostable DNA polymerase, which can withstand high temperatures without losing their ability to synthesise DNA, is the key component in the whole PCR technology. The *Taq* DNA polymerase and its derivatives resistant to high temperatures, which does not need to be replenished during the PCR amplification is the most popular.

***10.2.4 Magnesium ion concentration:*** Presence of free magnesium ($Mg^{++}$) is necessary for the performance of *Taq* ploymerase. In the absence of adequate free magnesium, *Taq* DNA polymerase is inactive. A final concentration between 1 and 1.5 mM is required for a PCR. However, Mg concentration has to be standardized for each PCR.

***10.2.5 Deoxyribonucleotides (dNTPs):*** Deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP) supplies the bases, the building blocks of DNA (A, C, G and T) during the PCR. Ample amount of dNTPs should be present in the PCR environment for a successful amplification.

Individual PCR components can be varied to optimise the PCR. Primer length and sequence are of critical importance in the design and optimization of PCR parameters.

***10.2.6 PCR amplification process:*** All the components of the PCR along with buffers, autoclaved-ultra pure water and optional addictives, are taken into a reaction tube (PCR vial) and subjected to PCR amplification process, comprising three major steps, denaturation, annealing and extension. All the three processes of PCR are carried out in the same vial, but at different temperatures. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time. The first step is the thermal **denaturation** where double stranded DNA template is heated to separate into single strands. This is done by heating the vial to 95º centigrade, for 15 seconds to 2 minutes. The second step is called the **annealing** where the vial is cooled approximately to 40-65° C, enabling the binding or annealing of the primers to the complimentary DNA strands. The third and final step is called the **extension** in which the temperature of the reaction is raised to the thermostable DNA polymerase, which is approximately 72-74° C for 1-2 minutes, to achieve the extension of primer sequence. The *Taq* polymerase adds the nucleotides to the primer sequences, eventually producing two new double stranded DNA fragments, which is a copy of the original double strand. This completes one cycle and the process of denaturation starts again with a return to 95° C, and the cycle can be repeated for 25-40 times. After the last cycle, one longer extension time of about 5 minutes is given to ensure that all reactions are completed. The machine used for the automated thermal cycling is the thermocycler or the PCR machine.

DNA sequence between the primer doubles after each cycle. Because both strands are copied during PCR, there is an **exponential** increase in the number of copies of the DNA. In thirty such cycles, PCR produces a billion-fold increase in the targeted section of DNA, amplifying otherwise undetectable DNA into enough material sufficient for diagnosis. This means, PCR can produce detectable amount of DNA from only few copies of pathogen, which cannot be detected by any other means.

***10.2.7 Detection:*** Once PCR generates microgram quantities of DNA, the same can be detected using simple techniques such as electrophoresis and fluorescent staining.

### 10.3 Modified PCR methods

Standard PCR has been modified to suit different purpose, producing different types of PCRs.

***10.3.1 Nested PCR:*** Nested PCR is simply a two step PCR where the amplified DNA from the first step PCR is used as the template in the second step, with a set of internal primers. The primers are complimentary to the sequences internal to the first set of primers. Using the nested primers, a second set of amplification is conducted. Nested PCR helps increase the specificity and sensitivity of PCR many folds.

***10.3.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)*:** In order to amplify the RNA, in the case of RNA viruses, the RNA is first converted to cDNA using reverse transcriptase enzyme. After reverse transcription, the PCR is conducted using cDNAs.

*Hot-start PCR*

This is helpful to reduce non-specific amplification.

***10.3.3 Touch-down PCR:*** Here the reaction starts at high annealing temperature and then the annealing temperature is reduced in steps, helps to reduce non-specific PCR products.

***10.3.4 Multiplex PCR:*** Simultaneous amplification of two or more unique targets of DNA sequences in the same specimen is carried out for pathogen analysis.

The above are few relevant ones with regard to diagnosis.

### 10.4. PCR optimization

Though PCR cycling conditions and composition of PCR components are by and large straight forward, each PCR needs optimization of PCR components such as quantities of magnesium, enzyme and template concentrations and ideal annealing temperature with reference to the primers used.

### 10.5 Contamination risks

The very high degree of sensitivity of PCR can pose problems of DNA contamination producing false positive results. Any contamination of the starting sample can result in the amplification of the wrong DNA sequence leading to a faulty diagnosis. Hence great care should be taken to keep the PCR laboratory clean for consistent and reliable diagnosis.

## 11. Conclusion

Use of nucleic acid based diagnostics are increasingly finding acceptance in the present day aquaculture, especially in viral disease management. PCR has enabled the amplification of viral sequences represented at extremely low levels within extracted nucleic acid pools (one copy of viral DNA per 70 000 cells), to levels detectable by gel electrophoresis and hybridization. Detection and screening of infected stocks from the rearing system with the help of gene probes and PCR has become a common management practice and offers great promise for the future of viral diagnostics and control. Nucleic acid based diagnosis are available for important viral diseases affecting farmed shrimps and fishes. Use of DNA/ RNA based diagnosis for bacterial and protozoan parasites are also finding fruitful results. Biotechnological companies are coming out with user-friendly DNA/RNA based diagnostic kits for the benefit of aquafarners. Use of these novel diagnostic tools is becoming the central part of disease prevention and management. These are also used for epidemiological studies for the better understanding of the pathogen. Further improvisation and sophistication of nucleic acid based diagnostics is in the offing with advent of DNA chips (DNA arrays), where diagnosis of multiple pathogens is possible in one single step.

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CHAPTER 12

# Potential Delivery Systems for Aquaculture Drugs and Nutraceuticals*

## Introduction

The growth of aquaculture industry in India has also led to intensification of the farming practices to maximize profits. Disease or threats have often accompanied this intensification, which besides impacting the farmed animals also creates stress on the environmental conditions. Thus, aquaculture drugs including nutraceuticals and antibiotics have become essential for fish farms to improve the growth performance and to treat disease outbreaks. These drugs are often administered to fish through feed or applied directly in culture systems. When a drug is applied in aquatic system, the level of drug in the serum of the animal sometimes increases beyond the therapeutic level and later declines to ineffective levels. This kind of 'saw tooth' drug kinetics is undesirable, especially when using toxic drugs at higher levels. This practical difficulty will be critical when a drug having a microlevel difference between effective therapeutic concentration and lethal concentration, is applied for control of diseases. Thus, a drug delivery system involving the use of biocompatible polymers becomes the focus of such research, which helps to maintain a high blood level of drugs or neutraceuticals over a longer period of time.

Drug delivery system is one of the fastest growing health sectors across the world. Sales of drugs incorporating drug delivery systems are increasing at an annual rate of 15 percent.

The major benefits of using drug delivery system are aimed at:

- Improving efficiency of drug under treatment
- Reducing toxicity and improving safety
- Improving bioavailability
- Optimizing pharmacokinitics
- Enabling rapid onset of action
- Improving chemical condition of aquatic ecosystem
- Improving therapeutic outcome
- Reducing environmental pollution

Over the years, there have been many conventional methods of administering aquaculture drugs and nutraceuticals, which include:

- Oral route
- Dip treatment

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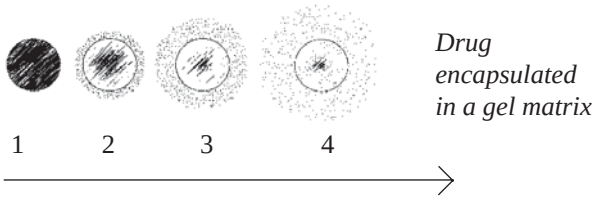
- Bath treatment
- Injection (intraperitonial, subcutaneous and intravenous).

However, there are different types of drug delivery systems used in pharmacology and therapeutics, which can be effectively used in aquaculture as drug delivery vehicles. They are bio encapsulation using the following preparations:

**1. Hydrogels**

A hydrogel is one that dries without significant collapse of the macroscopic structure and which absorbs water into its macropores without substantial swelling. In general, hydrogels are polymeric material having the ability to absorb and swell in water and retain a significant fraction (20 %) of water within its structure and remain insoluble. The equilibrium swelling degree or sorption capacity (swollen volume/dry volume) is the single most important property of a hydrogel with manipulation of the composition. Although the sorption kinetics does depend upon the gel composition, the sorption rate is roughly proportional to the equilibrium swelling degree and thus is also widely variable. The property of gels to form a film when dried can be used as a drug delivery system. Permeability to water, drugs, proteins and other solutes can also be varied over extraordinarily broad ranges, again, depending primarily upon the swelling degree or water content. *In vivo*, hydrogels can be designed to be biocompatible and biodegradable. The high water content of hydrogels also enhances their biocompatibility. The nature of the gel depends on the nature of cross-linker used, the polymer network, tenacity and crystallinity. Hydrophilicity and hydrophobicity are the chief means by which they control drug release. Hydrogels should be chemically inert and free of leachable impurities if they are to be used as controlled drug delivery formulations. They should be readily processable and with appropriate physical structure [*e.g.*, poly ethylene glycol]

***Diagrammatic representation of drug delivery system with the aid of encapsulation indicating that delivery system helps to bind the drugs/neutraceuticals for longer period of time.***




**2. Biodegradable gels**

Microspheres and microcapsules have been in vogue as one of the methods of drug delivery over four decades and in recent years a wide range of drugs and neutraceuticals such as antibiotics, steroid hormones, peptides, analgesic agents, immunomodulators and vitamins have been encapsulated.

**Biodegradable polymers for controlled delivery of proteins**

| Materials | Degradation mechanism |
|---|---|
| Starch | Amylase |
| Alginate | pH, Enzymes, Alginase |
| Collagen | Collagenase |
| Proteins | Enzymes, Proteases |
| Polyanhydrides | Hydrolysis |
| Polyesters (Polylactides) | Ester hydrolysis, Esterases |
| Poly (ortho esters) | Ester hydrolysis, Esterases |
| Polyiminocarbonates | Hydrolysis |
| Polycaprolactones | Hydrolysis |
| Polyamino acids | Enzymes, Proteases |
| Polyphosphazenes | Hydrolysis, Dissolution |

Various biodegradable gel-based drug vehicles in vogue are as follows:

**a. Liposomes**

When phospholipids are exposed to aqueous environment, closed bilayer structures, spontaneously form liposomes, which encapsulate part of the aqueous medium interior. The lipid bilayers mimic barrier properties of biomembranes. It is evident from biodegradable and non-toxic nature of phospholipid vesicles that these delivery systems are amenable to safe administration of drugs. Liposomes can alter the biodistribution of entrapped substances and protect the enclosed matrix from host-defense mechanisms. Thus, it can be used for target specific delivery of therapeutic drugs. In addition, liposomes are reported to reduce the toxicity of various chemotherapeutic agents and have demonstrated the ability to modulate immunogenicity of antigens. Recently, liposomes have proved as an effective delivery vehicle for gene therapy. Some of the phospholipids used in liposomes are dilauryl phosphatidyl choline (C 12:0), Dimyristic phosphatidyl choline (C 14:0), Dipalmitoyl phosphatidyl choline (C 16:0), Distrearoyl phosphatidyl choline (C 18:0), Egg phosphatidyl choline, etc. Another report clearly demonstrates the utility of novel non-ionic liposomal systems in facilitating transfer of growth hormone releasing peptide (GHRP-6) into and across deeper strata of skin following topical application.

**b. Chitosan**

Chitosan (CH) is a promising biodegradable drug vehicle used to deliver topical all trans retinoic acid (ATRA), which is a chemopreventive agent presently used agaist human skin cancers. CH gels help in sustained release of ATRA through skin, which were successfully prepared using gel entrapment technique. Example– 0.1 % of ATRA was dissolved in 5 % ethanol and 0.04 % butylated hydroxy toluene (BHT). Solutions up to 5 % CH were prepared by dissolving in glacial acetic acid. Higher concentrations were obtained by making slurry at 90$^{o}$ C, followed by addition of 1 % acetic acid. ATRA and CH gel slurry were mixed together to make thick slurry and applied transdermally against skin cancers.

### c. Shark gel

Reports suggest that gels made out of fresh meat of elasmobranchs, shark (*Scoliodon* sp.) using 1 % glacial acetic acid incorporated pelleted feed showed better stability and higher retention of L-ascorbate 2 tripolyphosphate Ca salt. This technique can be tried in the case of other aquaculture drugs and nutraceuticals.

### d. Alginates

Alginate is a water-soluble polysaccharide extracted from brown seaweed and is composed of alternating blocks of 1-4 linked α- L guluronic acid and β-D-mannuronic acid residues. Alginate is used extensively in the food industry as a thickener, emulsifier and a stabilizer. It is included in the group of compounds that are generally regarded as safe by the FDA.

**Composition of alginates obtained from different sources**

| Species | M content (%) | G content (%) | M/G ratio |
|---|---|---|---|
| *Macrocystis pyrifera* | 61 | 39 | 1.56 |
| *Ascophyllum nodosum* | 65 | 35 | 1.85 |
| *Laminaria digitata* | 59 | 41 | 1.45 |
| *Laminaria hyperborea* | 31 | 69 | 0.45 |
| *Ecklonia cava* | 62 | 38 | 1.6 |
| *Eisenia bicyclis* | 62 | 38 | 1.6 |

*M– Mannuronic acid ; G– Guluronic acid*

The gelation method for alginates involves dropping alginate solution into an aqueous solution of multivalent cations, which can be used as non-toxic reactants. Thus, alginates are the polymers of choice for cell encapsulation. The properties of the coating can be controlled by varying the parameters such as molecular weight, concentration of coating solution and coating time. In principle, multivalent cations can cause gelation of alginates. Barium and zinc have been used to form alginate gels. But magnesium will not form alginates. The affinity of cations to alginates is in the order: Pb> Cu>Cd>Ba>Sr>Ca>Co> Ni>Zn>Mn. It was found that alginates formed strong complexes with polycations such as chitosan, polyamino acids, polyethylenimine or polyacrylamide. Numerous reports have shown that the chemical structure, molecular size, gel forming kinetics and the cations have a significant effect on its functional properties including porosity, stability, swelling behaviour and bicomapatibility. Reports on the vaccination studies using calcium alginates against vibriosis in rainbow trout (*Onchorhynchus mykiss*) suggested that oral vaccination using alginate encapsulated attenuated *Vibrio anguillarum* was found to be effctive as a booster vaccination in combination with other vaccination methods as immersion or intraperitonial injection.

In aquaculture system, there is an urgent need to use similar biodegradable gels as a potential drug delivery vehicle along with various antibiotics after laboratory and field based studies on the efficacy and cost involved in administering the feeds and drugs.

### 3. Bioencapsulation

Bioencapsulation is a technique by which the drug or neutraceutical is introduced to the target animal through live feed. Thus, a direct application through the food chain using much smaller quantity has proven to be effective and safe for the environment. The quality of live feed depends on the content of essential fatty acids, eicosa pentanoic acid (EPA: 20: 5n-3) and docosa hexanoic acid (DHA: 22:6n-3). In contrast to the freshwater species, most marine organisms do not have the capacity to biosynthesize these essential fatty acids from lower chain unsaturated fatty acids, such as linolenic acid (18: 3n-3). *Artemia*, on hatching, feeds on particulate matter non-selectively. Thus lipid supplementation is widely applied in marine fish and crustacean hatcheries all over the world for many decades to enhance the nutritional value of live feeds. Reports suggested that 300 $\mu g\ g^{-1}$ of therapeutic mixture Trimetoprim: Sulphamethoxazole (1: 5) administration have yielded the serum antibiotic levels upto 20 $\mu g\ g^{-1}$ in Eurpoean seabass larvae. Similar results were observed in experiments conducted using an antibiotic, sarafloxasin in *Artemia franciscana*.

## Conclusion

The use of a tailor-made drug and nutraceutical delivery system ensures the slow release of drug or nutrients at constant levels to fishes and shellfishes irrespective of its life stages. It has a good potential to make a revolutionary change in the field of fish pharmacology and therapeutics in addition to the effective feed management procedures. This concept would also be critical from the environmental perspective by way of significant reduction in the residual nutraceuticals and antibiotics in the culture environmet.

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* * *

CHAPTER 13

# Hazards Analysis and Critical Control Points in Aquaculture*

## Introduction

Food safety issues associated with aquaculture products will differ from region to region and from habitat to habitat and will vary according to the method of production, management practices and environmental conditions. Foodborne parasitic infections, foodborne diseases associated with pathogenic bacteria, residues of agro-chemicals, veterinary drugs and heavy metal contamination have all been identified as hazard to aquaculture products. The origin of such food safety concerns are diverse, ranging from inappropriate aquacultural practices, environmental pollution and cultural habits of food preparation and consumption. Thus, with the increasing contribution of aquaculture to food fish supplies and to regional and international trade, proper assessment and control of any food safety concerns are becoming increasingly important.

## Hazards and risks

Inherently, in all human activities, including activities related to food production, there are hazards and risks, which may adversely affect human health and aquaculture is no exception. In this context it is particularly important to recognize that there is a fundamental difference between hazard and risk. A hazard is a biological, chemical or physical agent or condition in food, with the potential to cause harm. In contrast, risk is an estimate of the probability and severity of the adverse health effects in exposed populations, consequential to hazard(s) in food. Understanding the association between reduction in hazards that may be associated with food, and the reduction in the risk to consumers of adverse health effects is of particular importance in the development of appropriate food safety controls. The hazards identified are summarized below.

**Possible safety hazards in aquaculture products**

| ***Biological*** | | ***Chemical*** | |
|---|---|---|---|
| *Parasites* | Parasites of public health significance<br>Trematodes, Nematodes Cestodes | *Agro-chemicals* | Disinfectants, pesticides, herbicides, algicides fungicides, anti-oxidants (added in feeds). |
| *Pathogenic bacteria* | *Salmonella, Shigella*<br>*E.coli 0157, Vibrio cholerae*<br>*V. parahaemolyticus,V. vulnificus*<br>*Listeria monocytogenes*<br>*Clostridium botulinum* | *Veterinary drug residues* | Antibiotics, growth promoters (hormones), other feed additives from animal manures. |
| *Biological toxins* | Scrombrotoxin, Ciguatoxin | *Heavy metals* | Metals leached from soil, from industrial wastes, from sewage or animal manures. |

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***Biological Hazards***

A large number of fish species, both marine and freshwater, wild caught and cultured, can serve as a source of important parasites, which can be potential threat to human health. Some of these parasites may be transmitted to humans on consumption of raw or inadequately cooked fish. These infections are prevalent in only a few countries and mostly among communities where eating raw or inadequately cooked fish is a cultural habit. Generally, fish are the intermediary hosts of these parasites and man becomes the definitive host when the parasites are ingested. The principal human diseases caused by these parasites are trematodiasis, cestodiasis and nematodiasis.

Trematodes are by far the most important food safety hazard linked with fish and fishery products. Over 40 million people, mainly in eastern and southern Asia, are affected and more than 10 % of the world population is at risk of infection. Little or no information is available on the role of farmed finfish and crustaceans in the spread of diseases associated with the ingestion of these parasites.

Although seldom fatal, trematodes can cause morbidity and serious complications leading to death. The route of infection is through the ingestion of viable encysted metacercareae of parasites, which are present in the flesh of raw, inadequately cooked or minimally processed freshwater fish. The two major genera of importance for human health are *Clonorchis* and *Opisthorchis*, and of lesser importance *Paragonimus, Heterophyes* and *Metagonimus.*

Pathogenic bacteria can be introduced into fish ponds and watercourses by intentional or unintentional addition of human and animal waste. While it is understood that enteric bacteria and viruses rapidly die in well managed fish ponds, evidence suggests that enteric pathogens can survive and contaminate farmed products. Pathogens such as *Vibrio parahaemolyticus* and *V. cholerae* are unavoidable contaminants of cultured products and have been implicated in foodborne illnesses.

Chemical hazards can be present in aquaculture products through exposure to certain compounds used in the aquaculture system itself and by acute and chronic pollution of waterways or sources of water used. A broad range of chemicals are used in aquaculture-chemical fertilisers are widely applied to semi-intensively managed ponds in the tropics and sub-tropics to stimulate phytoplankton blooms. Such fertilisers may be either organic or inorganic in nature and are usually water soluble. They can be applied as individual compounds, or they may be blended to provide a mixed fertilizer containing two or more compounds. Although some of these compounds may be considered as hazard in their own right, in view of the concentrations and methods of use, they pose minimal risk to food safety in aquaculture products when used appropriately.

The use of antimicrobial agents in the aquatic environment is a cause for concern, both in terms of potential environmental impact and potential human health implications. The main concerns are associated with the uncontrolled sale and use of antibiotics as therapeutic agents, growth promoters and for increasing the efficiency of feed utilization in intensive and semi-intensive aquaculture systems. The hazards relate to veterinary drug residues and the development of antimicrobial resistance deriving from the use of antimicrobial agents.

As with all types of animal husbandry, infectious disease is an ever-present hazard in aquaculture, with the potential to cause major stock losses. To control such infectious diseases, the same range of strategies are employed as in other areas of animal production. The most effective approach is to prevent the introduction of diseases and pathogens, and for most areas where aquaculture is practiced, regulations are also in place, although with different standards of enforcement. Further, good husbandry is also essential to maintain good health of the farmed animals as also the environment.

However, limited data exists on the health risks associated with the use of antimicrobials in aquaculture, which preludes quantitative assessment of risk. Residues in products can be controlled by following recommended withdrawal times. The possible transfer of antimicrobial resistant pathogens to humans arising from the use of veterinary drugs in temperate aquaculture systems is thought to be low. However, this may be higher in tropical aquaculture, where antibiotics are used, because of higher temperatures and the survival of enteric human pathogens in fish ponds. In addition, antibiotics may inadvertently be added to fish ponds in integrated farming systems where they are either to treat diseased animals or as growth promoters.

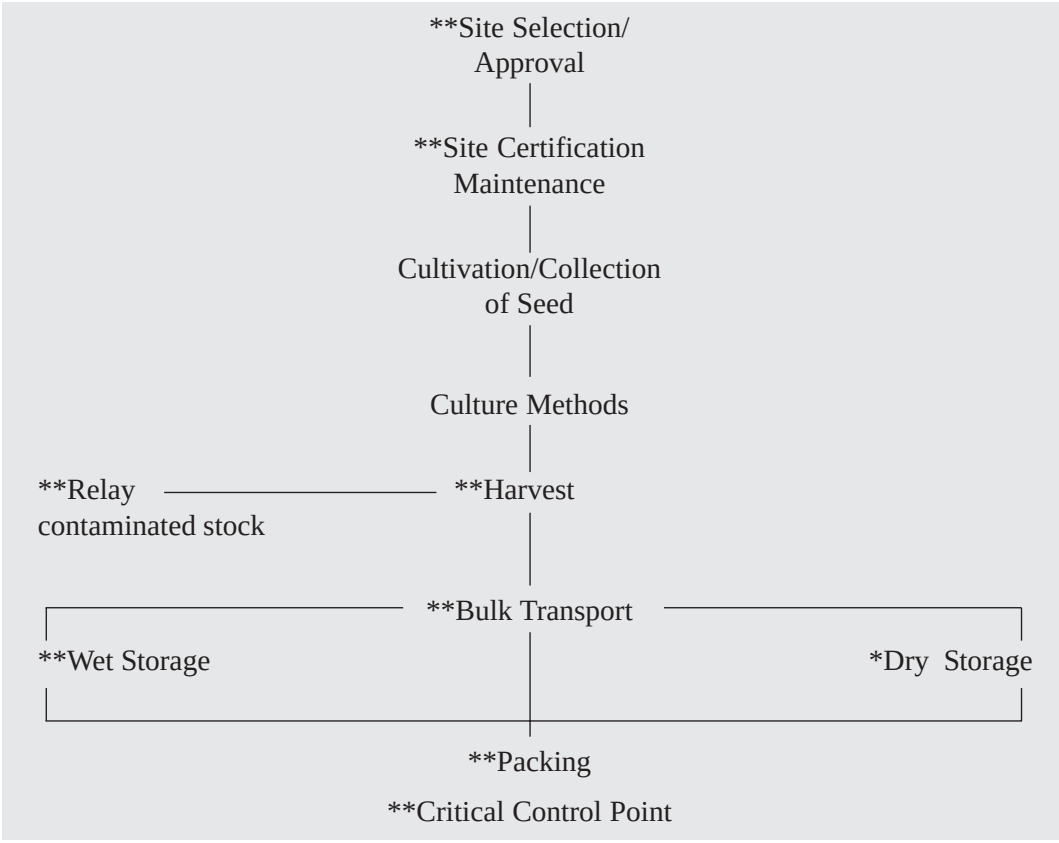
**Food safety from fish farm to table**

The role of the fish farmer is changing from merely raising fish to being an indispensable part of a chain for the production and delivery of safe, high quality products to the consumer. Hazards can be introduced into this food chain at the production stages on the farm and these can be spread during fish processing and preparation. Intervention strategies for assuring food safety are difficult to determine when microbial hazards cause human diseases but no diseases in fish, as in the case of some naturally occurring pathogenic *Vibrio* spp. or unavoidable contamination of fish ponds by *Salmonella* spp. in some aquaculture systems. Quantitative risk assessment is thought to be the most effective method of identifying contamination by microbial pathogens and this data could be used in making risk management decisions. Although this is an emerging discipline, data generated by microbial risk assessment can be used in the application of food safety assurance programmes based on the Hazards Analysis and Critical Control Point (HACCP) system. While the implementation of HACCP-based safety assurance programmes are well advanced in the fish processing sector, the application of such programmes at the fish farm, to enhance food safety, is in its infancy. The fish farming sector is not unique in this respect as there are few examples of the application of HACCP principles in animal husbandry because of the lack of scientific data regarding the appropriateness of on-farm control for pathogenic micro-organisms.

There is an international movement towards adoption of the HACCP system in the seafood sector, with such major markets as the European Union and North America introducing mandatory requirements for HACCP implementation during fish processing. Such requirements will impact on the aquaculture sector with respect to raw material standards and products moving in international trade. The introduction of HACCP-based food safety assurance programmes at fish farm level poses a major challenge to the aquaculture sector. With the current state of knowledge, the introduction is practical and is being applied in

some intensive aquaculture systems but not possible in small-scale fish farming that accounts for the bulk of global aquaculture production. An indicative 'Production Flow Chart' is depicted below.

**Production Flow Chart**




## Knowledge gaps and research needs

There is considerable need for information associated with this rapidly expanding sector of food production. Such gaps in knowledge will hinder the process of risk assessment and the application of appropriate risk management strategies with respect to food safety assurances and products from aquaculture.

## Biological hazards

### *Parasites*

Trematodes, and to a lesser extent cestode and nematode parasites, pose public health problems, particularly in Asia. There is no information available on the role of farmed finfish and crustaceans in the spread of diseases associated with the ingestion of these parasites.

Further research needs to be conducted on the epidemiology of trematode infections in cultured fish in relation to foodborne illness. Prior to establishing the comparative risk to human health from consumption of farmed and wild fish, it is necessary to determine the levels of trematode infection in farmed and wild fish and the influence of cultural practices of fish consumption. Research on the elimination of parasites in fish during processing should be given importance, particularly to determine the ability of the infective stages of these organisms to survive heat treatment. Freezing as a method to eliminate hazards associated with parasites in fish should be evaluated with respect to the possibility of allergic reactions and hypersensitivity. More work is needed to quantify the levels of infection of farmed fish by parasites and to evaluate the contribution of aquaculture products to foodborne trematode infections.

Stocking ponds with wild caught fry or allowing wild fish to enter ponds is a common practice in many areas. Epidemiological data should be obtained to evaluate the association of trematode infections in humans with such farming practices.

***Bacteria***

An unavoidable contamination of aquaculture products by foodborne pathogens, such as *Salmonella* and *Vibrio* can occur under some commercial farming conditions. Studies have to be made to apply molecular typing methods to distinguish between these and pathogens of human origin that may occur in products as a result of poor standards of hygiene during post-harvest handling and processing. There is also a need for methods to be developed for the detection of enteroviruses in aquaculture systems.

In view of the increasing importance of wastewater-fed aquaculture systems in developing countries, the potential for growth and survival of human enteric pathogens, particularly newly emerging strains of *Escherichia coli*, need to investigated.

In the light of international trade of food, microbiological risk assessment methods are required in many areas of food production. Such work was underway in the Codex Committee on Food Hygiene and recommended following Codex guidelines for the conduct of microbiological risk assessment for products from aquaculture.

The evaluation of such risks is constrained by the lack of quantitative data. Specific areas in aquaculture identified for the application of risk assessment methods are the use of moist animal-based feeds (*e.g.*, trash fish, bivalves and slaughterhouse waste), the ecology of *Listeria* in salmon aquaculture, the risks to public health from antibiotic resistant bacteria developing as a result of applying antibiotics in aquaculture, integrated animal husbandry/fish farming, and wastewater fed systems.

***Chemotherapeutants***

The following research is essential for the safe and effective use of chemicals in aquaculture:

- With respect to antimicrobial resistance, internationally agreed-upon and validated methods to determine the minimum inhibitory concentration (MIC) are needed.

Support from international bodies such as FAO and WHO would assist progress in this area.

- Agreed-upon and validated methods of residue analysis that do not impose excessive cost on consumers or producers are needed for compliance monitoring.
- Due to the limited number of approved veterinary medicines for use in intensive aquaculture in some countries, research is needed to enable products approved in one regulatory regime to be used in another without the cost of duplicate approval procedures.
- Certain types of integrated fish farming systems, where antibiotic-fed livestock are used, may pose a risk of antimicrobial resistance or unexpected residues in fish. The health implications of this type of artisanal production, combined with antimicrobial use is poorly understood and more information is needed before a proper assessment can be made.

**Conclusions**

- There is need for an integrated approach to controlling hazards associated with products from aquaculture; this requires close collaboration between the health, agriculture and aquaculture, food safety, and education sectors.
- Food safety assurance measures should be included in fish farm management programmes and should form an integral part of the fish "farm-to-table" food safety continuum.
- The food safety assurance measures should be based on the HACCP system.
- The risks to human health from chemicals used as fertilisers and water treatment compounds in aquaculture production are low.
- Risks from chemotherapeutants used in aquaculture are associated with residues in edible portions of fish flesh and these can be significant, especially in countries where the sale and use of these compounds are uncontrolled.
- There is the added risk of antimicrobial resistance developing in the bacterial flora of fish farms and of such antibiotic resistant bacteria entering the food chain.
- Pesticides required in aquaculture can pose food safety hazards and more information is needed on the types of compounds used. Studies should be conducted to determine if pond treatments with pesticides result in residue levels that are potentially harmful to human health.
- There is urgent need to raise the awareness of the fish farming community, especially small-scale rural subsistence farmers, to the concept of "Fish as Food" and to the impact of consumption of contaminated foods on human health.

- Education in the basic principles of food safety assurance should be integrated into existing regional and national training courses for aquaculture development and FAO/ WHO are urged to provide leadership in developing education material.
- Fish-borne trematodiasis is an important disease in some parts of the world, causing morbidity and serious health complications, which are sometimes fatal. Basic research is required on the survival of encysted metacercareae of these parasites in edible portions of fish during traditional processing and preparation; FAO/WHO are requested to co-ordinate research in this area.

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CHAPTER 14

# Guidelines for Regulations of Aquaculture Drugs in India*

## Introduction

Sustainability is an essential requirement for long-term viability of food-production sectors. The hallmark of success of any production process, agricultural or industrial, is determined by its capability to remain sustainable over the years. In aquaculture production, the concept of sustainability is being pursued for the last 2-3 decades, however little has been achieved, as the production processes are largely dependent on various extrinsic and intrinsic factors. Being a biological process that is carried out with greater dependence on natural environmental processes, aquaculture production has faced severe losses on many occasions. Such losses are largely due to the poor health of the aquatic environment and the animals being farmed. Therefore, maintenance of the aquatic environment and the health of the farmed animals is of utmost importance to make aquaculture production processes sustainable and less/minimum risk-prone. In this regard, the application of aquaculture drugs and chemicals assume significance as their use has to be fully regulated so that they perform beneficial functions and not lead to adverse impacts on the environment or be a health hazard for the consumers. This paper discusses some of the fundamental requirements and processes that need to be put in place to make the use of drugs and chemicals in aquaculture production processes absolutely safe for the environment as well as the consumers.

## Definition of aquaculture drugs and chemicals

Aquaculture drugs and chemicals are chemical or biological preparations or formulations which can be used in aquaculture systems to be administered to aquatic organisms at any stage of their life for management of their health and reproduction or applied directly to aquatic systems for management of the culture environment.

## Aquaculture medicine

Aquaculture medicine is a science to be practiced at field-level with the prime objective of maintaining the health of farmed aquatic animals and their environment. The past three decades have witnessed an ever-growing interest in this discipline world over and the developments, which are seen today, are the results of this awareness and growing requirements of the sector. The National Workshop on Aquaculture Medicine organized by

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the Centre for Fish Disease Diagnosis and Management (CFDDM), Cochin University of Science and Technology (CUSAT), Kochi, Kerala during January 2001 recognized the following subjects as part of the proposed disciplines of 'Aquaculture Medicine':

1. Diagnostics in Fish and Shellfish Health Management
2. Antimicrobials and Chemotherapeutics in Aquaculture Systems
3. Probiotics
4. Immuno-stimulants
5. Vaccines and Bacterins
6. Anesthetics
7. Hormones
8. Aquaculture Pharmacology
9. Population Medicine
10. Brood stock/Seed Certification and Quarantine
11. Aquaculture Grade Certification
12. Manufacture/Import and Marketing
13. Prescription/practice– Qualification of Practitioners in Aquaculture Medicine and Animal Health Management
14. Quality Control and Quality Assurance Programme
15. Intellectual Property Rights in Aquaculture Drugs
16. HACCP in Aquaculture

The objective of inclusion of the above 16 components under the subject of Aquaculture Medicine is to make the discipline comprehensive so that all the aspects of aquatic animal health management can be practiced at the field-level. Aquaculture drugs and chemicals, as health management tools, occupy a major component of aquaculture medicine because a variety of drugs and formulations have been introduced over the years in the farming sector. With rampant introduction of such drugs, chemicals and formulations from within and outside the country, the need for regulating their manufacture, import, marketing, prescription and practice has become an immediate necessity to ensure that the sustainability and ecological integrity of the aquaculture sector is maintained.

As on today, there is no legislation in the country to monitor and regulate the above activities. Bearing this in mind, the CFDDM organized a National Workshop on Aquaculture Drugs during January 2002 and constituted a 'High Level Committee' for drafting the recommendations for 'Aquaculture Drug Regulations' in the country. The first sitting of the committee was held on 17 January 2002 held along with the National Workshop on Aquaculture Drugs and the second meeting was held on 30 October 2002. The matter was subsequently debated at the national level for finalizing the recommendations that could be submitted to the Ministry of Agriculture, Government of India for enacting the regulations. In this context, the following part of the paper provides an overview on 'Aquaculture Medicine and Aquaculture Drug Regulations for Aquatic Animal Health Management in India'.

## Aquaculture medicine– An overview

### 1. Diagnostics

Rapid diagnosis of diseases, an essential requirement for effective disease management, can be antibody based or nucleic acid based. With the introduction of hybridoma technology, monoclonal antibodies are now available to counter various pathogens of importance in aquaculture sector. While polyclonals have their own importance in terms of low cost and better sensitivity, one has to be extremely careful in selecting such tools concerning their sensitivity and cross reactivity. Since antibodies are generated based on the antigenic epitopes of the pathogens, the antibodies used for the development of the diagnostics should be the one raised against the native pathogenic strains, as the antigenic profile may vary between the strains of native and alien species. Moreover, handy tools such as Dot Blot, ELISA (Enzyme-linked Immuno-sorbent Assay), Latex Agglutination Test, etc., also have to be developed and made available commercially at field level. A major breakthrough in DNA based diagnostics is the introduction of diagnostic PCR in the detection of pathogens in very low numbers, particularly viruses.

### 2. Antimicrobials and chemotherapeutics

One of the greatest achievements of medical science is the capability achieved in the management of human pathogens either by prophylaxis or therapy. In the same line, antimicrobials and chemotherapeutics have an important role to play in aquaculture in the management of both primary and opportunistic pathogens. Unlike the use of chemotherapeutics in human beings and terrestrial animals, the application of similar compounds in aquaculture has great environmental impacts, especially with respect to the aquaculture environment. It has to be understood that for a healthy aquaculture system, a healthy microbial flora is essential in the soil-water system, which should not be impaired at any cost. The environmental impacts of management chemicals and chemotherapeutics have to be fully known before preparing for an application mode. With respect to those chemotherapeutics applied through diet, their residual effect(s) in the animal body should be known before resorting to their application. The antimicrobials and chemotherapeutants are classified as follows:

a) General management chemicals, such as those meant for water and sediment quality management
b) Weedicides
c) Antiviral preparations
d) Antibacterials, including antibiotics
e) Antifungal compounds
f) Antiprotozoal, antimetazoal and anthelmintic compounds

Several feed additives can also be included under this category. Considering the potential threats of environmental impact and residual effects in the animal body, the High Level Expert Committee on Aquaculture Drug Regulation (hereinafter referred to as HLEC-ADR)

brought out a set of criteria to be stipulated in every such commercial product meant for aquaculture application. On this basis, every such product should be accompanied with the following information:

- Product name
- Chemical name and structure
- Active ingredient grade/strength
- Mode of action
- Mode of application and recommended preparations required for applying in water/aquatic system
- Side effects in animals, if any
- Effects in the environment and human beings, if any
- Metabolism in the animal body and mode of excretion
- Residues in animals and environment (withdrawal period)
- Shelf life and storage
- Uses and statutory warning

**3. Probiotics**

The word 'Probiotic' is derived from the Greek meaning 'for life' and has had several different meanings over the years. It was believed that beneficial organisms could balance the intestinal environment, prevent the growth of pathogenic bacteria, consequently improving health and prolonging life. The administration of beneficial organisms to animals first started in the year 1929, and the name probiotics was introduced in 1974 when the production of bacterial feed supplements began on a commercial-scale.

In aquaculture, probiotics can be divided into two broad categories, *viz*., gut probiotics and pond probiotics. This classification is based on the fact that the probiotics are live microbial supplements that are believed to have a positive role in improving the animal health. This change can be direct, by improving the quality of the microbial flora of intestine and enhancing the quality of the water sediment systems. Some of the proposed modes of action could be prevention of initiation of colonization by potential pathogens by antagonism or competitive inhibition, or by acting as an immune-stimulant. There is no proper scientific data available on the ability of the probiotics to colonize the gut in aquatic animals. This implies that the gut probiotic application must be carried out in continuous mode to ensure that viable cells are available in the intestine continuously.

The pond probiotics can be called as bioremediators too, as the purpose of this group of organisms is to bioremediate the pond system, making it congenial for aquatic animals. It is the process of treatment of any waste by use of microbes that breaks down the undesirable substances. The groups of microbes that are categorized under bioremediators are detritivorous bacteria, nitrifiers and photosynthetic sulphur bacteria.

Efficacy of probiotic organisms depends on their capability to multiply in pond conditions and colonize for long or short periods. In this context, there is a need to validate the usefulness

of probiotics that are imported, in terms of their sustainability. Such alien organisms are likely to vanish within no time, necessitating frequent additions. This strongly highlights the need for developing indigenous probiotics to suit regional specifications.

Probiotics, in general, have attracted international attention and several categories of preparations are available. Ironically, very little scientific information is available on any of these preparations. Considering the fact that a strict quality assurance has to be provided by the manufacturers, the HLEC-ADR formulated the following criteria to satisfy the requirements of granting permission to market them:

- Product name
- Species
- Strains and strain numbers (including source)
- Composition
- Status- native or introduced; if introduced, name of the agency that cleared introduction; if indigenous, basic scientific data on field trials
- Viable count
- Mode of action
- Mode of application and recommended preparation required for applying in aquatic system
- Impact on the environment and known side effects in animals and human beings, if any
- Fate of the organism in the animal body/aquatic environment
- Uses, viability, storage and statutory warning

**4. Immuno-stimulants**

By definition, an immune-stimulant is a chemical, drug, stressor or action that elevates the non-specific defense mechanism or the immune-suppressor. Vaccination is probably the best method of specific immune-stimulation providing increased resistance against a specific antigen/pathogen. Non-specific immune-stimulation refers to a condition in which the immune response is stimulated to respond towards a variety of antigens. As the aquatic animals encounter a variety of pathogens in the environment, highest priority is given to non-specific stimulation of immune system using immune-stimulants.

The immune-stimulants may either be grouped by function or by origin. A few important ones are glucan preparations, lipopolysaccharides, levamisole, vitamin C and E. These substances act in various ways *viz.*, modify the cell membranes, stimulate the macrophage lymphocytes, natural killer cell activities, activate the complement system, acting as carriers/ vehicles, reservoirs/depots, including inflammation and cytokines production. However, extensive work is required to accurately establish the time, dose, period, cost, stability and side effects of each of the substances in a particular growth stage of fish and shellfish species.

Even before the completion of basic works, several commercial products have started pouring into the aquaculture sector. Therefore, the HLEC-ADR proposed certain specifications to be attached to each commercial product. These are:

- Product name
- Source (chemical/biological)
- Composition (with concentration of each)
- Active ingredients
- Basic scientific data on field trials
- Mode of action
- Mode of application and recommended preparations required for applying in aquatic system
- Impact on the environment and known side effects in animals and human beings, if any
- Metabolism in the animal body/aquatic environment
- Uses, shelf life and storage and statutory warning.

Every manufacturer is expected to furnish the above details of the commercial preparation.

**5. Vaccines and bacterins**

Vaccines are preparations of antigens derived from pathogenic organisms, rendered non-pathogenic by various means and aimed at stimulating the immune system in order to increase the resistance to disease from subsequent infection by pathogen. Protective antigens can be isolated, characterized, purified and synthesized biochemically or using DNA recombinant technology. For bacterin preparation, whole cell bacterial antigens responsible for inducing protective response need to be maintained during the inactivation steps, which may be formalin treatment or heat inactivation. Among the different kinds of vaccines, bacterial biofilm vaccine and DNA vaccines are areas to be explored as novel prophylactic tools.

Even though crustaceans are not supposed to have memory and specificity like vertebrates, recent studies suggest the existence of the above in rudimentary form. This gives much hope in the development of appropriate immunization programmes against potential and opportunistic pathogens of the cultivable species.

Considering the importance of vaccines as possible prophylaxis, the HLEC-ADR has put forth the following specifications to be declared by the manufacturers of every such product:

- Product Name
- Composition
- Species strain and strain number, status– native or introduced, if introduced, name of the agency that cleared introduction, if indigenous, basic scientific data on field trials
- Type of vaccine
- Mode of application and recommended preparations required for applying in aquatic system
- Safety and toxicity in animal
- Fate of the vaccine and its side effects in the animal body/aquatic environment, if any
- Shelf life and storage, uses and statutory warning.

## 6. Anesthetics

Appropriate handling of animals at various life stages is essential in aquaculture practices. Inappropriate handling can cause stress and physical damage to the animal, which in turn makes the animal susceptible to attack by bacteria and fungi. In shellfish culture, transport of broodstock from wild to the hatchery or seed from hatchery to grow outs also forms one of the routine activities. The use of anesthetics makes these activities easier, reducing the chances of physical damage and stress to the animals.

The major recognized anesthetics are MS – 222, Benzocaine, Phenoxyethanol, Quinaldine, Chlorobutanol, Tertiary butyl alcohol, Propanidid, Diazepam, Butanol, Ether, Vernol, Thiouracil, Paraldehyde, Phenobarbital sodium, Chloral hydrate, etc. However, much research is required on the mode of administration, side effects, etc.

As anesthetics are chemicals, the criteria stipulated by manufacturers of anesthetics as commercial products falls within the purview of chemicals and the same set of stipulations as given for chemicals in aquaculture need to be followed.

## 7. Hormones

Hormones in aquaculture are of two categories– growth and reproductive. The major hormones recognized in aquaculture are somatotropin, 17- a - methyl testosterone, oestradiol, ovulation inducing drugs such as gonadotropic releasing hormone and its analogous, dopamine antagonists such as dompenidone, pimozide and serotonin.

In crustaceans, reproductive or growth hormones as products are not yet available. However, a lot of information is available on the endocrine system of crustaceans, which secrete peptides, steroids and sesquiterpenoids that play vital roles in reproduction and moulting.

The mammalian hypothalamic leuteinizing releasing hormone (LHRH) and its super-active analogues are found to have the best applications in aquaculture. Carps and catfishes with ripe gonads were found to readily respond to injections of the LHRH analogue in micro quantities. Subsequently, GnRH from teleostean species were characterized and super-active analogues were synthesized. Thus a commercial preparation named ‘Ovaprim’ containing salmon GnRH analogue and a dopamine antagonist Dompexidone produced by M/s Syndel Laboratories, Canada have become favourite with fish breeders all over the world. A similar preparation named ‘Ovatide’ is now manufactured and marketed by M/s Hemmo Pharma, Mumbai and is reported to give comparable results. A Hungarian product ‘Ovopel’ is now at the testing stage in India.

Like hormones, pheromones have potential application in breeding and aquaculture. They may be used in accelerating ovarian recrudescence, enhanced milt production as well as selective stimulants in composite fish culture, even without affecting the non-target species. Since they are externally active, their delivery will eliminate the stress associated with the hormone or drug administration. These chemicals may also be employed as artificial baits, selective attractants, growth stimulants and inhibitors of aggression and cannibalism.

Considering the availability of a couple of commercial products and the possibility of having more in the coming years, the HLEC-ADR stipulated the following criteria for every such product:

- Product name
- Composition
- Active ingredient
- Grade/strength
- Mode of action
- Mode of application
- Side effects in animals, if any
- Effects in the environment and human beings, if any
- Metabolism of the drug in the animal body and mode of excretion
- Residues in animals and environment (withdrawal period)
- Shelf life and storage, uses and statutory warning

**8. Aquaculture pharmacology**

Aquaculture pharmacology is yet another branch in aquaculture medicine, which needs attention from all quarters. This includes drug formulation and design, drug delivery systems, pharmacodynamics and pharmacokinetics.

**9. Population medicine**

Epidemiology or population medicine is now being increasingly recognized as an important weapon that can be used in the fight against diseases. Epidemiology in its strict sense is the study of diseases in population in its natural setting, which can be of great advantage over laboratory studies that may not be easily extrapolated to field conditions.

**10. Brood stock/seed certification and quarantine**

Introduction of exotic diseases due to trans-boundary movement of aquatic animals can have several social, economic and ecological consequences. Effective quarantine is a must to minimize the chances of introduction of exotic pathogens of concern associated with the import of live aquatic animals and aquatic animal products. There is very little likelihood to eradicate an exotic disease once it gets established, especially in aquatic animals. Therefore, development and implementation of an effective, transparent and practical system of brood stock/seed certification and quarantine is the need of the hour.

**11. Aquaculture grade certification**

Any medicine meant for aquaculture application must get an aquaculture grade certification. According to the 'aquaculture grade' proposed by the HLEC-ADR, a drug or chemical or biological formulation will qualify for aquaculture grade certification, if it meets the criteria set out under each category (*e.g.* drugs, chemicals, probiotics, immune-stimulants, etc.) in the aquaculture drug regulations. The aquaculture grade certification would be given by a

competent authority in the Government in consultation with a technical committee constituted for the purpose.

**12. Manufacture/import and marketing strategies**

Any licensed manufacturer under the Drugs and Cosmetics Act, 1940 can manufacture aquaculture grade drugs/chemicals/biological formulations. A technical committee of aquaculture specialists set up by the Government can advise on the clearance of import of drugs and chemicals based on the criteria set forth. The existing Expert Committee on Aquaculture Drug Regulations can send its recommendations to the Ministry of Agriculture, Government of India. The existing provisions in the Drugs and Cosmetic Act, 1940 will also govern the marketing of aquaculture grade drugs and chemicals.

**13. Prescription/practice– qualification of practitioners of aquaculture medicine**

With the emergence of diseases in aquaculture system, several pseudo-specialists have emerged in this field, who are ill-qualified and ill-equipped to face the challenges and guide farmers in proper direction. On several occasions, they serve as agents of drug manufacturers too. Considering the importance of professionals in this field, qualification of such categories of practitioners of aquaculture medicine has been fixed by the HLEC-ADR, which is as follows;

> *"An aquaculture medicine practitioner should have a sound knowledge of the aquatic environment, anatomy and physiology of aquatic animals, medicine and pharmacology. A graduate or postgraduate from Fisheries/Veterinary College and other traditional University with proper qualification can become a registered practitioner after undergoing appropriate course in Aquaculture Medicine".*

An appropriate curriculum for Aquaculture Medicine has been framed by the HLEC-ADR and is under finalization before it is submitted to the Indian Council of Agricultural Research (ICAR) and the University Grants Commission (UGC) for their consideration for implementation in various educational institutions. The Expert Committee has also suggested the formation of Fisheries Council of India similar to Medical Council and Veterinary Council of India for registration of practitioners of aquaculture medicine.

Candidates who wish to undertake this programme should either have Bachelor of Fisheries Science degree of any Agriculture University or Master's Degree in Aquaculture, Mariculture, Marine Biology or Fishery Science of any traditional University. Agricultural Universities can offer this as Masters of Fisheries Science (M.F.Sc.) as approved by ICAR and traditional Universities as Masters in Technology (M.Tech.) after obtaining due concurrence from AICTE and UGC. An apex body should regulate the curriculum, content and conduct of the programme. The apex body should consist of members drawn from Deemed Fisheries University, ICAR, AICTE, UGC, Ministry of Agriculture, Aquaculture Authority, National Centre for Aquatic Animal Health (NCAAH), and Central Government Fisheries Institutions. The committee shall work under the Chairmanship of the Deputy Director General (Fisheries), ICAR, and coordinated by Aquaculture Authority and convened by NCAAH.

The HLEC-ADR also proposes the following curriculum for M.F.Sc./M.Tech. in Aquaculture Medicine and Aquatic Animal Health Management.

**Semester - I**

1. Dynamics of aquaculture environment
2. Pathogens in aquaculture systems
3. Parasites in aquaculture systems
4. Fish and shellfish disease

Practicals

1. Physical, chemical and biological quality of aquaculture environment
2. Identification of pathogens

**Semester - II**

1. Diseases of fin fishes
2. Diseases of shell fishes
3. Disease diagnosis
4. Pharmacology of aquaculture medicine

Practicals

1. Disease diagnosis
2. Aquaculture pharmacology

**Semester - III**

1. Prophylaxis: Probiotics, immunostimulants and vaccines
2. Therapy in aquaculture
3. HACCP in aquaculture and aquaculture impact assessment
4. Sociology, psychology and ethics in aquaculture medicine

Practicals

1. Fish and shell fish immunology
2. Prophylaxis and therapy

**Semester - IV**

Full time dissertation

### 14. Quality control and quality assurance programme

Presently, no quality standards are prescribed for any of the drugs used for aquaculture; therefore the quality may vary from batch to batch and thereby the performance too. This is a serious issue and it is proposed that quality control and quality assurance of aquaculture drugs and biological formulations would be governed as per the provision under the Drugs and Cosmetic Act, 1940. As described earlier, the criteria for quality of aquaculture drugs have been specified and the manufacturer is expected to follow the norms. The contents of such products have to be declared. To monitor the quality of the products, quality control

and quality assurance laboratories have to be set up in different parts of the country. There must also be adequate machinery to check periodically the quality of each product.

**15. Intellectual property right issues involved in aquaculture drugs**

Several companies and manufacturers would like to protect the intellectual property rights and are hesitant to declare the composition of the contents of the product/formulations. However, for the regulation of quality control, declaration of the content and composition of the product is essential. To satisfy both the requirements, the Committee felt that in the case of novel drugs and formulations, patenting have to be carried out, which will prevent copying of any formulations. In such situations, the IPR issues relevant to aquaculture grade drugs and biological formulations will be governed by the existing regulations for human and veterinary grade drugs and the relevant provisions in the Indian Patent Act, 1970.

**16. HACCP in Aquaculture**

The Hazard Analysis and Critical Control Points (HACCP) system is being increasingly implemented in food production cycle. This is more relevant in the marine food production system. The HLEC-ADR discussed the provisions of HACCP in aquaculture production system and recommended that the provisions of HACCP pertaining to the use of drugs, chemicals, antibiotics, etc., should be fully incorporated in to the aquaculture production cycle.

**Conclusion**

Aquaculture production has become increasingly complex with many issues, within and outside the production processes that need to be addressed and overcome. Over the years, the Indian aquaculture sector has not been adequately following the code of practices, disregarding the requirements of sustainability as also food safety. Drugs and chemicals in aquaculture have to be designed taking into consideration the fact that they are being applied in a biological environment, which will respond in varying degrees. Therefore, aquaculture medicine has to be treated as a serious branch of science to be practiced at field level. The aquaculture drug regulations, when enforced will ensure that the farming community works under a set framework, with required checks and balances. It is once again emphasized that if aquaculture production has to be stabilized and made sustainable, fish farmers have to be trained to work in a disciplined manner and contribute to the overall growth of the aquaculture sector in the country.

**End Note**

This article is open for discussion, debate and critical evaluation. Those who would like to interact with the authors, may do so through email.

## Further reading

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# Author Index



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# Subject Index

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**List of antibiotics and other pharmacologically active substances banned* for use in shrimp aquaculture**

| Sl. No. | Antibiotics and other pharmacologically active substances |
|---|---|
| 1 | Chloramphenicol |
| 2 | Nitrofurans including: Furaltadone, Furazolidone, Furylfuramide, Nifuratel, Nifuroxime, Nifurprazine, Nitrofurantoin, Nitrofurazone |
| 3 | Neomycin |
| 4 | Nalidixic acid |
| 5 | Sulphamethoxazole |
| 6 | Aristolochia spp. and preparations thereof |
| 7 | Choloroform |
| 8 | Chlorpromazine |
| 9 | Colchicine |
| 10 | Dapsone |
| 11 | Dimetridazole |
| 12 | Metronidazole |
| 13 | Ronidazole |
| 14 | Ipronidazole |
| 15 | Other nitroimidazoles |
| 16 | Clenbuterol |
| 17 | Diethylstilbestrol (DES) |
| 18 | Sulfonamide drugs (except approved Sulfadimethoxine, Sulfabromomethazine and Sulfaethoxypyridazine) |
| 19 | Fluroquinolones |
| 20 | Glycopeptides |

* Aquaculture Authority, Ministry of Agriculture, Government of India.

## Maximum permissible residual levels for fish and fishery products*

| | Substance | Maximum permissible residual levels (in ppm) |
|---|---|---|
| **A.** | **Antibiotics and other pharmacologically active substances** | |
| 1. | Chloramphenicol | Nil |
| 2. | Nitrofurans including: Furaltadone, Furazolidone, Furylfuramide, Nifuratel, Nifuroxime, Nifurprazine, Nitrofurantoin, Nitrofurazone | Nil |
| 3. | Neomycin | Nil |
| 4. | Nalidixic acid | Nil |
| 5. | Sulphamethoxazole | Nil |
| 6. | Aristolochia spp. and preparations thereof | Nil |
| 7. | Chloroform | Nil |
| 8. | Chlorpromazine | Nil |
| 9. | Colchicine | Nil |
| 10. | Dapsone | Nil |
| 11. | Dimetridazole | Nil |
| 12. | Metronidazole | Nil |
| 13. | Ronidazole | Nil |
| 14. | Ipronidazole | Nil |
| 15. | Other nitroimidazoles | Nil |
| 16. | Clenbuterol | Nil |
| 17. | Diethylstilbestrol (DES) | Nil |
| 18. | Sulfonamide drugs (except approved Sulfadimethoxine, Sulfabromomethazine and Sulfaethoxypyridazine) | Nil |
| 19. | Fluroquinolones | Nil |
| 20. | Glycopeptides | Nil |
| 21. | Tetracycline | 0.1 |
| 22. | Oxytetracycline | 0.1 |
| 23. | Trimethoprim | 0.05 |
| 24. | Oxolinic acid | 0.3 |

| | | | |
|---|---|---|---|
| **B.** | **Substances having anabolic effect and unauthorised substances** | | |
| | 1. | Stilbenes, stilbene derivatives and their salts and esters. | Nil |
| | 2. | Steroids | Nil |
| **C.** | **Veterinary drugs** | | |
| | 1. | Antibacterial substances, including quinolones | Nil |
| | 2. | Ante helminitic | Nil |
| **D.** | **Other substances and environmental contaminants** | | |
| | 1. | Organochlorone compounds including PcBs | Nil |
| | 2 | Mycotoxins | Nil |
| | 3. | Dyes | Nil |
| | 4. | Dioxins | Nil |
| **E.** | **Pesticides** | | |
| | 1. | BHC | 0.3 |
| | 2. | Aldrin | 0.3 |
| | 3. | Dieldrin | 0.3 |
| | 4. | Endrin | 0.3 |
| | 5. | DDT | 5.0 |
| **F.** | **Heavy Metals** | | |
| | 1. | Mercury | 1.0 |
| | 2. | Cadmium | 3.0 |
| | 3. | Arsenic | 75 |
| | 4. | Lead | 1.5 |
| | 5. | Tin | 250 |
| | 6. | Nickel | 80 |
| | 7. | Chromium | 12 |

* Aquaculture Authority, Ministry of Agriculture, Government of India.

# Glossary

**ACTH** – Hormone produced by the anterior pituitary gland that stimulates the adrenal cortex.

**Acute infection** – Clinical manifestation of disease, which occurs over a short period of time.

**Aetiologic agent** – The primary organism (usually a microorganism) responsible for changes in host, leading to disease.

**Agglutinin** – Specific factors present in sera, which agglutinate or clump organisms or particulate protein matter.

**Agglutinin titre** – The concentration of antiboby at greatest dilution capable of causing specific clumping or agglutination of cell particles.

**Algae bloom** – A high density or rapid increase in abundance of algae.

**Alkalinity** – The ability of a mineral solution to neutralize hydrogen ions; usually expressed as equivalent of calcium carbonate.

**Ammonia nitrogen** – Also called total ammonia. The summed weight of nitrogen in both the ionized (ammonium, $NH_4^+$) and molecular ($NH_3$) forms of dissolved ammonia ($NH_4$ - N plus $NH_3$-N). Ammonia values are reported as N (the hydrogen being ignored in analyses).

**Ammonia** – The gas $NH_3$ is highly soluble in water. Ionized ammonia ($NH_4^+$) is relatively non-toxic to fish while the un-ionized form is extremely toxic. The percent of the total ammonia in un-ionized form is a function of pH and temperature.

**Anaemia** – A condition characterized by deficiency of haemoglobin, packed cell volume and/or erythocytes.

**Analgesic** – A collective term for any member of the diverse group of drugs used to relieve pain. Analgesic drugs include the nonsteroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, narcotic drugs such as morphine, and synthetic drugs with narcotic properties such as tramadol. Other classes of drugs not normally considered analgesics are used to treat neuropathic pain syndromes; these include tricyclic antidepressants and anticonvulsants.

**Anorexia** – Loss of appetite.

**Antennal gland** – Excretory pores at the base of the antennae (also known as kidney gland, excretory organ and green gland).

**Antibiotic resistance** – The capability of a microbe to evade destruction by an antibiotic.

**Antibody** – A highly specific protein molecule produced by plasma cells in the immune system in response to a specific chemical substance; antibodies function in antibody-mediated immunity.

**Antigen** – A substance that elicits an immune reaction in a vertebrate host.

**Anti-inflammatory** – Medicine intended to reduce inflammation.

**Antimicrobial agent** – An agent that kills microorganism or inhibits their growth.

**Antimicrobial drugs** – The compounds such as dyes and synthetic or organic substances used in the treatment of microorganisms.

**Antiseptic** – Chemical agents applied to tissue to prevent infection by killing or inhibiting pathogens.

**Aseptic (sterile)** – Free from infection/infective agent(s).

**Astringent** – Drug that causes contraction of body tissues and canals.

**Atrophy** – Decrease in the amount of tissue, or size of an organ after normal growth has been achieved.

**Attenuated virus** – A weakened variant virus that emerges during successive transfers of the virus in tissue cultures; used in immunization because of its reduced virulence; some times called "Live virus".

**Autolysis** – Enzyme induced rupture of cell membranes, either as a normal function of cell replacement or due to infection.

**Avirulent** – An infectious agent which does not cause any pathology.

**B and T Lymphocytes** – Type of white blood cells. Lymphocytes make up a quarter to a third of the white blood cells. Then there are two types of lymphocytes, B and T cells. The T lymphocytes help the B cells make antibodies as part of the immune response.

**Bacteria** – Unicellular microorganisms that multiply by cell division, the nuclear material of which are not contained within a nucleus, typically have a cell wall; may be aerobic or anaerobic, motile or non-motile, free-living, saprophytic or pathogenic.

**Bacterial Kidney Disease (BKD)** – A disease of trout and salmon caused by the bacterium *Renibacterium salmoninarum*. The disease is systemic but derives its name from off-white bacterial lesions in the kidney. In later stages many organs may become affected and the body cavity may become filled with fluid.

**Basophilic** – Acidic cell and tissue components staining with basic dyes such as hematoxylin. Chromatin and certain secretory products in stained cells appear blue or purple.

**Bioassay** – A qualitative or quantitative procedure that uses susceptible organisms to detect toxic substances or pathogens.

**Bioavailability** – The portion of a nutrient in a diet that is absorbed and used for one or more biological functions.

**Biopsy** – Examination of tissues or liquids from the living body to determine the existence or cause of a disease.

**Blood Dyscrasias** – A general term which is used to describe any abnormality in the blood or bone marrow's cellular components, such as low white blood cell count, low red blood cell count, or low platelet count.

**Broodstock** – Sexually mature male and female animals used to produce fertilized eggs.

**Buffers** – Chemicals capable of taking up or giving up hydrogen ions and sustains pH within a narrow range.

**Calcium Carbonate** – A relatively insoluble salt, $CaCO_3$, the primary constituent of limestone and a common constituent of hard water.

**Canthaxanthin** – A careteniod pigment used to colour the flesh of fish through inclusion in the feed. Also called carophyll red.

**Carbon Dioxide** – A colorless, odorless gas, $CO_2$, resulting from the oxidation of carbon-containing substances; highly soluble in water. Toxic to fish at high levels. Toxicity to fish increases at low levels of oxygen. May be used as an anesthetic.

**Carbonate** – The $CO_3^-$ ion, or any salt formed with it, such as calcium carbonate, $CaCO_3$.

**Carrier** – Organism harboring specific pathogen without manifesting symptoms, capable of transmitting the infection.

**Catecholamine** – Any of a group of chemicals including epinephrine and norepinephrine that are produced in the medulla of the adrenal gland.

**Chemical synapses ("synapses")** – Specialized junctions through which cells of the nervous system signal to one another and to non-neuronal cells such as muscles or glands. Synapses are circuits in which the neurons of the central nervous system interconnect. They are thus crucial to the biological computations that underlie perception and thought. They also provide the means through which the nervous system connects to and controls the other systems of the body.

**Chemotaxis** – Movement by a cell or organism in reaction to a chemical stimulus.

**Chemotherapeutant** – A chemical used to treat a disease.

**Chitin** – A linear polysaccharide found in the exoskeletons of arthropods, the cell walls of most fungi, certain algae and the cyst walls of ciliates.

**Chitinolytic** – Chitin degrading bacteria/fungi, producing enzymes capable of breaking down chitin component of arthropod exoskeletons.

**Chronic** – Long-term infection, which may or may not manifest clinical signs.

**Clinical** – Pertaining to actual observations.

**Collagen** – The principal proteinacous substance in connective fibers.

**Corticosteroids** – Hormones produced by the adrenal cortex that have, among other things, anti-inflammatory properties. For example, corticosteroids interfere both with the activity of phospholipases (thereby compromising the production of arachidonic acid and, subsequently, of the eicosanoids) and the expression of inducible genes that are quantitatively key to the production of certain kinds of pro-inflammatory mediators. For example, corticosteroids interfere with the expression of the gene that encodes cyclooxygenase-2, without which the production of prostaglandins by inflammatory cells is greatly reduced.

**Cortisol : Cortisol is a small molecule that functions as a chemical messenger (hormone).** When brain stimulates its release, glands found on top of the kidneys secrete cortisol into the blood. Principally a stress hormone, it is synthesized in response to stress and subsequently modifies several diverse parameters (blood sugar levels, blood pressure, etc.), empowering the body to react.

**C-reactive protein** – A by-product of inflammation; a globulin that is found in the blood in some cases of acute inflammation.

**Cuticle** – The protein structure of arthropods consisting of an outer layer (epicuticle), an underlying exo-cuticle, calcified endo-cuticle and membranous layer containing chitin (except in epi-cuticle).

**Cyst** – A dormant stage of a free-living or parasitic organism.

**Cytology** – The study of cells, their origin, structure and function.

**Cytopathic effect** – Pathological changes in cells.

**Decalcification** – The process of removing calcareous matter.

**Diagnosis** – Determination of the nature of a disease.

**Diazepam** – A tranquilizer used to relieve anxiety and relaxes muscles; acts by enhancing the inhibitory actions of the neurotransmitter GABA; can also be used as an anticonvulsant drug in cases of nerve agent poisoning.

**Disease** – Any deviation from the normal structure or function of any part, organ, or system of the body that is manifested by characteristic signs and symptoms.

**Disinfection** – Procedures that destroy infectious agents of diseases of aquatic animals, hatcheries, fish farms and contaminated objects.

**DNA (Deoxyribonucleic Acid)** – Genetic material of all cellular organisms and most viruses, containing the bases adenine, guanine, cytosine and thymine, and phosphate.

**DNA gyrase** – A topoisomerase that relieves supercoiling in DNA by creating a transient break in the double helix.

**DNA probes** – Segments of DNA labeled to indicate detection of homologous segments of DNA in samples of tissues or cultures.

**Domperidone** – A specific blocker of dopamine receptors. It speeds gastrointestinal peristalsis, causes prolactin release and is used as antiemetic tool in the study of dopaminergic mechanisms.

**ELISA (Enzyme Linked Immunosorbent Assay)** – Assay used to detect antigen or antibody.

**Endemic** – Usually prevalent in a population or geographical area at all times.

**Enteric redmouth disease** – A disease caused by *Yersinia ruckeri*. Also called as "Hagerman redmouth", "salmonid blood spot".

**Enzootic** – Present in a population at all times but, occurring only in small number of cases.

**Eosinophilic** – Basic cell and tissue components staining with acidic dyes such as eosin. Stained cells appear pink to red.

**Epidemiology** – Study of the factors determining and influencing the frequency and distribution of disease and its causes in a defined population for development of prevention and control strategies.

**Epithelium** – The layer of cells covering the surface of the body and gastrointestinal linings.

**Epizootic** – Affecting a large number of animals within an area at the same time that is rapidly spreading.

**Epizootiology** – Study of factors influencing infection by a pathogenic agent.

**Erosion** – Destruction of the surface of a tissue, material or structure.

**Erythrocytes (Red Blood Cells)** – The cells that carry oxygen.

**Euthanasia (Greek, "good death")** – Practice of killing a person or animal, in a painless or minimally painful way, for merciful reasons, usually to end suffering.

**Exoenzyme** – Extra cellular enzyme released by cells or microorganisms.

**Exopthalmia** – Abnormal protrusion of the eyeballs.

**Exoskeleton** – The chitin and calcified outer covering of crustaceans which protects the soft-inner tissues.

**Filtration** – Passage of a liquid through a filter, by gravity, pressure or vacuum.

**Fin rot** – A bacterial disease caused by *Pseudomonas fluorescens*. Other synonyms are bacterial tail rot, hemorrhagic septicemia.

**Fingerlings** – Small or juvenile fish, not of marketable size.

**Fixation** – Preservation of tissues in a liquid that prevents protein and lipid breakdown and necrosis; the specimen is hardened to withstand further processing; and the cellular and sub-cellular contents are preserved in a manner close to that of the living state.

**Fixative** – A fluid that prevents denaturation and autolysis of tissues.

**Fouling** – Colonisation of hard substrates by free-living organisms.

**Free Radical** – A molecule with an unpaired electron. Because they have a free electron, such molecules are highly reactive.

**Fungi** – Single-celled or multinucleate (coenocytic) organisms that live by decomposing and absorbing the organic material.

**Furunculosis** – A salmon disease caused by the bacteria *Aeromonas salmonicida*.

**GMO** – An organism that has been modified by gene technology; an organism that has inherited particular traits from an organism (the initial organism), being traits that occured in the initial organism because of gene technology; or anything declared by the regulations to be a genetically modified organism, or that belongs to a class of things declared by the regulations to be genetically modified organisms.

**Gonadotropins** – Gonadotropins are the hormones produced by the pituitary gland that control reproductive function. They include follicle stimulating hormone and luteinizing hormone.

**Gram's Stain** – A differential stain, used for differentiating bacteria.

**Granulocytes** – (a) In vertebrates – granular leucocytes (oesinophils, basophils and neutrophils, (b) In invertibrates – haemocytes with or without esinophilic cytoplasmic granules (granular or agranular respectively) and with a higher nucleoplasm- cytoplasm volume ratio than hyalinocytes. Granular granulocytes are often called phagocytes, however, agranular granulocytes and hyalinocytes also have phagocytic capabilities.

**Gross signs** – Signs of disease visible to the naked eye.

**Haematopoietic tissue** – Tissue involved in the formation of blood cells. In penaeid shrimp, the tissue surrounding the lateral arterial vessels, secondary maxillipeds and epigastric tissues; in fish, it is spleen.

**Haemocyte** – Blood cells of crustaceans.

**Haemolymph** – Cell-free fraction of the crustacean blood containing proteins and nonproteinaceous defensive molecules.

**Haemorrhage** – (a) Internal or external bleeding in vertebrates and invertebrates caused by rupture of blood vessels. Capillary haemorrhaging within a tissue may cause tissue displacement. (b) Uncontrolled loss of haemocytes in invertebrates due to tissue trauma or epithelial rupture.

**Hardness** – The ability of water to neutralize soap, due to the presence of cations such as calcium and magnesium; usually expressed as parts per million equivalents of calcium carbonate. Refers to the calcium and magnesium ion concentration in water on a scale of very soft (0-20 ppm as $CaCO_3$), soft (20 - 50 ppm), hard (50 - 500 ppm) and very hard (55+ ppm).

**Hemorrhagic septicemia** – A fish disease caused by *Aeromonas hydrophila*. Also called by infectious dropsy, infectious abdominal dropsy or red sore.

**Hepatopancreas** – The digestive organ of crustacea, composed of ducts and tubules, which secrete digestive enzymes and also involved in the release of metabolic by-products and microbial wastes.

**Histology** – The study dealing with structure, composition and function of tissues.

**Histopathology** – Study of structural and functional changes in tissues and organs caused by a disease.

**Husbandry** – The practice of cultivating the land or raising stock.

**Hydrophilic** – Having a strong affinity for water; tending to dissolve in, mix with, or be wetted by water. Describes a substance that attracts, dissolves in, or absorbs water.

**Hydrophobicity** – The property of being water-repellent; tending to repel and not absorb water; a measure of how insoluble (undissolvable) a given molecule is when in water or other polar liquids.

**Hyperplasia** – An abnormal increase in size of a tissue or organ due to an increase in number of new cells.

**Hypertrophy** – Abnormal enlargement of cells due to irritation or infection by an intracellular organism.

**Hypervitaminosis** – In fish because fat – soluble vitamins are stored in tissues, excessive intake of these vitamins can cause health problems. Occurrence of this disease requires massive dietary vitamin levels.

**Hyphae** – Filamentous cells of fungi.

**Hypoxin** – Oxygen deficiency in tissues or organs.

**Immunity** – Protection against infectious disease conferred either by the host immune response generated by immunization or previous infection or by other non-immunologic factors.

**Immunization** – Protection against disease through exposure to pathogen to induce defense system enhance subsequent immune response to exposure to the same pathogen.

**Immunoassay** – Technique using the antigen-antibody reaction to detect and quantify the antigens and antibodies.

**Immunofluorescence** – An immuno-histochemical technique using antibody labeled with a fluorescent dye.

**Immunoglobulin** – A group of proteins containing light and heavy molecular weight chains linked by disulphide bonds, usually produced in response to antigenic stimulation.

**Immunoglobulin (Ig)** – A family of proteins made up of light and heavy molecular chains linked together by disulphide bonds; usually produced in response to antigenic stimulation.

**Immunostimulant** – An immunostimulant stimulate the specific immune system when given along with an antigen, or it may stimulate the nonspecific immune system when given alone.

**Immunostimulation** – Enhancement of defense response.

**Inclusion body** – Non-specific bodies found within the cytoplasm or nucleus of a cell, frequently found during viral infections (Cowdry type-A body, Polyhedrin Inclusion).

**Infection** – Invasion and multiplication of an infectious organism within host tissues that may be clinically benign or resulting in cell or tissue damage.

**Infectious** – Capable of causing infection.

**Interferons** – A family of antiviral agents secreted *in vivo* and *in vitro* by virus – infected cells; they protect neighboring cells and thus the organism from super infection by virus

**Interleukins** – Interleukin is the generic name for a group of well-characterized cytokines that are produced by leukocytes and other cell types. They have a broad spectrum of functional activities that regulate the activities and capabilities of a wide variety of cell types. They are particularly important as members of the cytokine networks that regulate inflammatory and immune responses.

**Juveniles** – Young stages of animals, usually between the post-larval stages up to the time they first become sexually mature. They are generally hardy at this stage.

**Lesions** – Abnormal changes in tissues or body functions.

**Lethargy** – Abnormal drowsiness.

**Leukopenia** – An abnormal decrease in the number of white blood cells, often reducing immune system function.

**Lordosis** – An exaggerated forward, convex curve of the lumbar spine.

**Lymphocytes** – A type of white blood cell. Three important kinds of lymphocytes are T-cells, B-cells, and Natural Killer Cells. T-cells attack and destroy virus-infected cells, foreign tissue and cancer cells; B-cells produce antibodies that help destroy foreign substances; Natural Killer cells destroy cancer cells and virus-infected cells.

**Lymphoid organ** – An organ situated between the anterior and posterior stomach chambers that connect the sub-gastric artery to the anterior aorta, via a mass of interconnected tubules.

**Lysin** – An antibody, which causes cell lysis, or a bacterial toxin, which lyses (ruptures) cell membranes.

**Lysozyme** – An enzyme that degrades peptidoglycan by hydrolyzing the â (1-4) bond that joins N-acetylmuramic acid and N-acetylglucosamine.

**Macrocytic -** Any abnormally large cell. The opposite of macrocytic is microcytic (an abnormally small cell). An abnormally large red blood cell. Folic acid deficiency is one cause of macrocytic anemia.

**Macrophage** – A large amoeboid blood cell, responsible for phagocytic inflammation, antibody and cytotoxin production in vertebrates.

**Melanin** – Dark brown-black pigment of indole quinone having enzyme inhibiting properties. It forms part of the primary defense mechanism against cuticle and epidermal damage in many crustaceans.

**Melanisation** – Abnormal deposits of melanin.

**Microbicide** – Any agent detrimental to, or destructive of, the life of microbes on bacterial organisms.

**Microorganism** – Microscopic organisms including, viruses, bacteria, and fungi. Microscopic protozoa, algae can be also included under microooorganisms.

**Microsporidians** – A kind of sporozoan.

**Molluscicidal** – The ability to kill mollusks.

**Monoclonal antibodies** – Identical antibody molecules produced by cloning of the antibody producing cell that is responsive to a single antigenic epitope.

**Monocytes** – A type of white blood cell.

**Moulting** – The shredding of the exoskeleton to facilitate growth of internal soft tissues (Crustacea).

**Moulting** – The shedding of the exoskeleton to permit growth of internal soft-tissues also referred to as ecdysis.

**Mutagen** – A chemical or physical agent that causes mutation.

**Mycosis** – Disease resulting from infection by a fungus.

**Natural Killer Cells** – A type of white blood cell that contains granules with enzymes that can kill tumor cells or microbial cells. Also called large granular lymphocytes (LGL).

**Necrosis** – Progressive irreversible changes indicative of cell death, affecting groups of cells or part of a tissue or an organ, caused by degradative enzymes.

**Nephrons** – Nephrons are the functional units of the kidney. Nephrons are composed of a Bowman's capsule, a series of ducts and closely associated blood vessels. Nephrons working under hormonal control precisely regulate the osmotic concentration of blood and thus all body fluids. Nephrons in the kidney along with the collecting ducts produce hypertonic urine, this helps mammals conserve water and remove nitrogenous wastes (urea) from the body. Antidiuretic hormone (ADH) promotes the reabsorbtion of water. Cells in the brain called osmoreceptor cells monitor blood osmotic concentration. These cells stimulate production of ADH and stimulate thirst.

**Neurotransmitter** – Transmits nerve impulses across a synapse.

**Neutrophile** – Microorganisms that grow best at neutral pH range between pH 5.5 and 8.0.

**Nutraceuticals** – They are not vitamins or minerals, but highly specialized food derivatives that serve essential needs of the body. It is otherwise called as "a nutraceutical is any food or food ingredient considered to provide medical or health benefits, including the prevention and treatment of disease."

**Oedema** – Presence of abnormally large amounts of fluid in the intercellular spaces of the body.

**Opportunistic pathogen** – An organism capable of causing disease only when the host's immunity is lowered by factors such as adverse environmental conditions, or other factors.

**Oxidative burst** – One of the earliest events which occurs following challenge with a potential pathogen is the generation of active oxygen species (AOS). These AOS are predominantly the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) which arise from successive one electron reductions of molecular oxygen. AOS generation appears to occur irrespective of the type of eliciting pathogen; bacterial, fungal, or viral. AOS generation is localised to the point of infection and appears to occur primarily in the apoplasm even if an intracellular pathogen is involved, *e.g.* a virus.

**Parasitaemia** – A condition in which parasites are present in the blood.

**Parasite** – An organism which lives upon or within another living organism.

**Passage** – Successive transfer and culture of a pathogen through a series of experimental animals, tissue culture, or synthetic media.

**Pathogen** – An infectious agent capable of causing disease.

**Pathogenicity** – The ability to produce pathological changes or disease in a host.

**Pathognomonic** – Sign or symptom that is distinctive for a specific disease or pathological condition.

**Pathology** – Deals with the nature of disease, and the structural and functional changes in tissues and organs that are caused by a disease.

**Peptidoglycan** – A large polymer composed of long chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues. The polysaccharide chains are linked to each other through connections between tetrapeptide chains attached to the N-acetylmuramic acids. It provides much of the strength and rigidity possessed by bacterial cell walls.

**Pereiopods** – Thoracic appendages in crustaceans, referred as walking legs.

**Phagocytosis** – Uptake of particles (infective agents) by a cell by the process of invagination of its cell membrane.

**Pharmacodynamic** – Concerned with the response of living tissues to chemical stimuli, that is the action of drugs on the living organism in the absence of disease.

**Pharmacokinetics** – The study of what happens to a drug after it is applied to an animal. This discipline is still at an early stage for aquaculture species, and most of the available information has been developed in the last 10 years.

**pKa** – The pKa or ionisation constant is defined as the negative logarithm of the equilibrium coefficient of the neutral and charged forms of a compound. This allows the proportion of neutral and charged species at any pH to be calculated, as well as the basic or acidic properties of the compound to be defined.

**Platelets (Thrombocytes)** – A blood cell that assists in blood clotting. Patients are at risk to bleed if the platelet count is less than 50,000.

**Pleopod** – Small appendages referred as swimming legs in aquatic crustaceans.

**Polymerase Chain Reaction (PCR)** – A process by which nucleic acid sequences are replicated.

**Polyvalent vaccine** – A recombinant organism into which has been cloned antigenic determinants from a number of different disease-causing organisms. In other words, a vaccine that immunizes against more than one antigen.

**Prevalence** – Percentage of animals in a sample infected by a specific disease.

**Prophylactic** – Action or chemotherapeutant administration to healthy animals to prevent infections.

**Prophylaxis** – Prevention of disease.

**Prostaglandins** – A group of compounds derived from unsaturated 20-carbon fatty acids, primarily arachidonic acid, via the cyclooxygenase pathway. They are extremely potent mediators of a diverse group of physiological processes.

**Pseudotuberculosis** – A fish disease caused by *Photobacterium damselae*.

**$Q_{10}$** – Symbol for the increase in rate of a process produced by raising the temperature by 10° C; rate of contraction of an excised heart approximately doubles for every 10° C (*i.e.*, Q10 = 2).

**Quarantine** – Holding or rearing of aquatic animals under conditions, which prevent their escape, and the escape of any pathogens they may be carrying into the surrounding environment. This usually involves sterilization/disinfection of all effluent and quarantine materials.

**Redox potential** – Redox is short for Reduction Oxidation. Redox is a measure of a systems capacity to oxidize material. Otherwise Redox is a classification category of chemical reactions. Redox reactions are fewer and slower by comparison. In reduction-oxidation reactions, one chemical species loses, another gains electrons. Redox potential is a value (number) defining how much gaining/losing a system might do; an oxidizing environment (one we want) has a positive value. Redox is measured indirectly as the ability of an aquatic system to conduct electricity, in millivolts (mV, 1/1000 of a volt). Hence the term Redox potential is otherwise called as Oxidation-Reduction Potential.

**Reflex** – An automatic instinctive unlearned reaction to a stimulus.

**Reservoir** – A passive host or carrier that harbors pathogenic organisms, without having disease itself, and serves as a source from which other individuals can be infected.

**Resistance to disease** – Capacity of an organism to control the pathogenic effects of an infection. Heavy sub-clinical infections suggest resistance in an animal.

**Scoliosis** – A lateral curvature of the spine.

**Sea lice** – Small crustaceans (Lepeophtheirus) that attach themselves to salmon and feed on their flesh, producing unsightly blemishes. Efforts by a fish to rub the lice off can lead to infections.

**Septicaemia** – A systemic disease associated with the presence and persistence of pathogenic microorganisms or their toxins in the blood.

**Sterilization** – Any physical or chemical process, which kills or destroys all contaminating organisms.

**Streptococcosis** – (*Lactococcus garviae* infecton, A B C D , Streptococcosis (*L. garviae* infection in particular) has been associated with serious economic loss in cultured Japanese amberjack since 1974 . Clinical signs include exophthalmia and distended abdomen, hemorrhaging in the eye, in the opercula and at the base of the fins, and necrotic ulcer formation on the caudal peduncle. The causative agent is a Gram-positive spherical or oval bacteria less than 0.2 μm in diameter, which occur in pairs. The organisms are well growing on TSA, BHIA, THBA, or blood agar. The colonies are small (0.5-1.0 mm diameter), yellowish, translucent, rounded and slightly raised.

**Stress** – Disturbance in an organism's physiology by any of the adverse physical, internal or external factors.

**Sub-clinical infection** – An infection with no apparent signs and symptoms of disease.

**Synaptic Transmission** – The communication from a neuron to a target (neuron, muscle, or secretory cell) across a synapse. In chemical synaptic transmission, the presynaptic neuron releases a neurotransmitter that diffuses across the synaptic cleft and binds to specific synaptic receptors. These activated receptors modulate ion channels and/or second-messenger systems to influence the postsynaptic cell. Electrical transmission is less common in the nervous system and as in other tissues, is mediated by gap junctions. Hormone produced principally in response to physical or psychological stress and secreted by the adrenal glands.

**Syndrome** – Clinical signs manifested together indicate a distinct disease or abnormality.

**Systemic infection** – Infection involving the whole body.

**Translocation** – Chromosomes are actually very sticky and very fragile. Sometimes during mitosis the chromosomes break and then join themselves back together. Occasionally, they do not join back where they originally broke. This results in a condition called a balanced translocation.

**Transpeptidation** – The reaction that forms the peptide cross-links during peptidoglycan synthesis.

**Ulcer** – Excavation of the surface of an organ or tissue, involving sloughing of necrotic inflammatory tissue.

**Uropods** – Terminal appendages in crustacean underlying the telson that form the 'tail fan'.

**Vaccine** – Preparation of an antigen from whole or parts of a pathogen, which is used to enhance the specific immune response of the susceptible host.

**Virion** – Individual viral particle containing nucleic acid, DNA or RNA and a protein coat (capsid).

**Virology** – Branch of microbiology concerned with the study of viruses.

**Virulence** – Degree of pathogenicity caused by a pathogen, as indicated by the severity of the disease produced and its ability to invade the tissues of the host.

**Virus** – A minute infectious agent, characterized by lack of independent metabolism and by the ability to replicate only within living host cells.

**Y-organ (Ecdysal gland)** – An organ involved in the production of the moulting hormone ecdysone.

**Zymosan** – An insoluble preparation of yeast cell has been shown to activate macrophages via TLR 2. Upon stimulation with zymosan, TLR 2 is recruited to the phagosome and signals the production of TNF-alpha through the NF-kB pathway. TLR 2 cooperates with CD14 in response to zymosan, as suggested by the high levels of NF-kB observed in TLR2+/CD14+ macrophages exposed to the ligand compared to the levels obtained in TLR2+/CD14- macrophages. Furthermore, TLR6 and TLR2 were reported to coordinate macrophage activation by zymosan.

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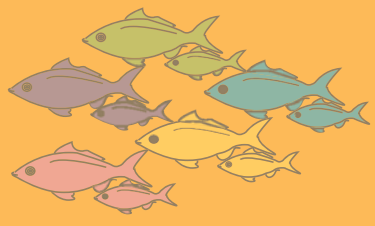
NCAAH: WWW.ncaah.org
Google Scholar: https://scholar.google.co.in/citations?user=sxRlEFgAAAAJ&hl=en
Research Gate: https://www.researchgate.net/profile/Bright_Singh

Dr Yugraj Singh Yadava, 52, has 30 years of experience in fisheries and aquaculture. He has served as Scientist with the Indian Council of Agricultural Research (1976 -1992) and later moved to fisheries development and administration (1993- till date). As Fisheries Development Commissioner to the Government of India, Dr Yadava played a key role in setting up of the Aquaculture Authority in early 1997 and also served as its first Member Secretary. After moving to Chennai in August 2000 to head the Bay of Bengal Programme of the Food and Agriculture Organization of the United Nations, Dr Yadava was once again given the charge of Member Secretary, which he is currently holding along with his position in the BOBP. A well-known face in the national and international fisheries sector, Dr Yadava has published more than 130 papers in books, journals and magazines.



The Aquaculture Authority is a regulatory body constituted as per the directives of the Supreme Court judgement delivered on 11 December 1996 in Writ Petition No 561 *(Civil)* of 1994. Set-up by the Ministry of Environment and Forests under Section 3 (3) of the Environment *(Protection)* Act, 1986, the Authority functions under the administrative control of the Government of India in the Ministry of Agriculture. The Authority is headed by a retired Judge of the Madras High Court with experts drawn from the fields of aquaculture, pollution control and environment protection and representatives from the Ministries of Agriculture, Commerce and Industry and the Environment and Forests as Members. The headquarters of the Authority are located at Chennai, Tamil Nadu.

The primary objective of the Authority is to regulate shrimp farming in the coastal areas of the country. To promote sustainable shrimp farming, the Authority provides guidelines to shrimp farmers on subjects such as adoption of improved technology for increasing production and productivity in traditional and improved traditional systems of shrimp farming, setting up of effluent treatment systems in shrimp farms above 5 hectares and application of Precautionary Principle. The Authority brings out a quarterly Newsletter *"Aquaculture Authority News"*, which is also available on the web site *(http://aquacultutre.tn.nic.in)* of the Authority.

National Centre for Aquatic Animal Health (NCAAH) was established by Cochin University of Science and Technology (CUSAT), Cochin on 18th January 2000, with the mandate to carry out research, education and extension in aquatic animal health for strengthening the aquaculture industry. The NCAAH is headed by Coordinator and assisted by an Advisory Board. Vice Chancellor of CUSAT is the Patron of the Centre.

NCAAH undertakes programmes with external funding and also with the revenue generated from the aquaculture industry. The Centre functions with technical manpower as Research Associates, Post Doctoral Fellows, Senior and Junior Research Fellows and Project Assistants. The short and long-term objectives, achievements and information on the activities of the Centre are avaliable on its website *http: www.ncaah.org*

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