ADVANCED BIOTECHNOLOGY PROCESS 'THE BIOREMEDIATION' TO RESTORE THE HEALTH OF AQUACULTURE POND ECOSYSTEM

Abstract

Aquaculture is concerned with 'the propagation and rearing of aquatic organisms under complete human control involving manipulation of atleast one stage of an aquatic organism's life before harvest, in order to increase its production'. Fish catches from the marine environment have been steadily declining in many parts of the world due to over-exploitation and pollution, many people are turning to aquaculture to improve the food production and to contribute for economic development. Aquaculture, in India, has made encouraging progress in the past two decades producing significant quantities of food, income and employment. Aquaculture, particularly, tiger shrimp *Penaeus monodon* culture, has extensively been practiced all along coastal regions of India. Increased production is being achieved by expansion of culture areas and the use of modern methods. This development of aquaculture in our country has led to not only severe disease problems but also alteration of the quality of our natural habitats through increased effluent discharges from aquaculture systems, which contains high quantities of hither-to-non-existent materials of both organic and inorganic forms. Since recent past it has been observed that the sustainable development of aquaculture sector can be achieved by adopting eco-friendly aquaculture practices by minimizing impact on the surrounding environment. To maintain healthy ecosystem in aquaculture ponds and hatchery tanks bioremediation is the best biotechnology process. Many researchers has been demonstrated that the pathogens can be eliminated or minimized through this bio-control process and hence can achieve good yield by maximizing both survival rate and growth rate and by minimizing the disease problems in aquaculture systems.

Key words: Aquaculture; Over-exploitation; Pollution; Penaeus monodon; Sustainable development; Eco-friendly; Bioremediation; Probiotics; Enzymes; feed additives; Water Quality; Disease control.

Introduction

During the past 20 years, aquaculture industry has been growing tremendously, especially that of marine fish, shrimps and bivalves in addition to freshwater fish and prawn. Penaeid shrimps are among the most important and extensively cultured crustaceans in the world (> 60 countries). In 2002, the world's shrimp farmers produced an estimated morethan 1.0 million metric tons of whole shrimp and feed mills around the world produced approximately 1.5 million metric tons of shrimp feed.

Shrimp culture all over the world has been frequently affected by viral and bacterial diseases inflicting huge losses and annual losses due to disease has been estimated to be morethan US $ 5 billions. Pathogenic microorganisms implicated in these outbreaks were viruses, bacteria, algae, fungi and protozoan parasites. Although diseases pose a serious threat to penaeid shrimp aquaculture, the production of these highly valued crustaceans continue to grow. For preventing and controlling diseases, particularly in aquaculture, the best method is the improving health of culture organisms and elimination of pathogens by improving aquatic environment. Thus, how to improve the ecological environment of aquaculture has become the focus of attention of international aquaculture. Now, researchers have proved the use of probiotic bacteria in aquaculture to improve water quality by balancing bacterial population in water and reducing pathogenic bacterial load. Researchers are increasingly paying more attention to this new approach (ecological aquaculture), and have made considerable headway. In addition to this each year, globally, microbial pathogens cause millions of cases of foodborne disease and result in many hospitalizations and deaths (Jordan Lin, C.T., et al., 2005).

Competition for survival can be strong, even on a scale invisible to the human eye. Indeed, aquaculture pond bacteria so small that 10,000 fit on a pinhead are subject to the same law of evolution as that Darwin documented on the Galapagos, that is, survival of the fittest or competitive exclusion. Competitive exclusion is one species out-competing another in a natural search for habitat dominance. As old as time, competitive exclusion is providing science with a neat new means of addressing health and environmental challenges now. The use of competitive exclusion for improving a specific ecology is called "probiotics" (Parkar. 1974). Probiotics therapy intentionally introduces strains of beneficial bacteria in order to replace bad bacteria. The first application of probiotics in aquaculture seems relatively recent (Kozasa, 1986), but the interest in such environment-friendly treatments is increasing rapidly.

Aquatic animals are quite different from the land animals for which the probiotic concept was developed, and a preliminary question is the pertinence of probiotic applications to aquaculture. Man and terrestrial livestock undergo embryonic development within an amnion, whereas the larval forms of most fish and shellfish are released in the external environment at an early ontogenetic stage. These larvae are highly exposed to gastro-intestinal microbiota-associated disorders, because they start feeding even through the digestive tract is not yet fully developed (Timmermans, 1987), the though the immune system is still incomplete (Vadstein, 1997). Thus, probiotic treatments are particularly desirable during the larval stages.

The transience of aquatic microbes is legitimate the extension of the probiotic concept to living microbial preparations used to treat aquaculture ponds. Moriarty (1997, 1998) proposed to extend the definition of probiotics to microbial water additives.

In 1991, Porubcan reported on two attempts at bacterial treatments to improve water quality and production yield of Penaeus monodon. (1) Pre-inoculated with nitrifying bacteria decreased the amounts of ammonia and nitrite in the rearing water. This treatment increased shrimp survival (Porubcan, 1991a) . (2) The introduction of Bacillus spp. In proximity to pond aerators reduced chemical oxygen demand, and increased shrimp harvest (Porubcan, 1991b). Moriarty (1998) noted an increase of shrimp/prawn survival in ponds where some strains of *Bacillus* spp. were introduced. The actual data of Moriarty (1998) showed the inhibitory activity of *Bacillus* spp. against luminous vibrio sp. in pond sediment, but the effect on shrimp/prawn survival might be due either to a probiotic effect, or to an indirect effect on animal health. For instance, the degradation of organic matter by Bacillus spp. might improve water quality.

The probiotic treatments may be considered as methods of biological control, the so-called "biocontrol" that termed the limitation or the elimination of pests by the introduction of adverse organisms, like parasites or specific pathogens. Maeda et al. (1997) proposed to designate as biocontrol the methods of treatment using "the antagonism among microbes through which pathogens can be killed or reduced in number in the aquaculture environment". Another terminology should designate the applications of nitrifying bacteria that are related to the bioremediation concept. This concept refers to the treatment of pollutants or waste by the use of microorganisms that break down the undesirable substances (Fig.1).

Sugita et al. (1998) isolated a strain of Bacillus sp. that was antagonistic to 63% of the isolates from fish intestine. Pathogenic strains of *Vibrio* or *Aeromonas* have been targeted in most in vitro tests. Treatment with *Lactobacillus brevis* and lactic acid reduced the load of *Vibrio alginolyticus* in the *Artemia* culture water (Villamil, L., et al., 2003). Some bacteria are antagonistic to viruses (Kamei et al., 1987, 1988; Direkbusarakom et al., 1998), and they may be efficient for the biocontrol of viral diseases (Maeda et al., 1997) and some other probiotics are effective in controlling bacterial diseases in fishes (Aubin, J., et al., 2005).

The resistance cause by probiotics to infections to shrimp, prawn and fish is due to – Bacterial antagonism, bacterial interference, barrier effect, colonization resistance and competitive exclusion in addition to improving biodegradation of waste organic matter through nitrogen cycle.

To best determine, which bacteria make good probiotics for aquaculture production, scientists scoop up quantities of water from healthy habitat in the ocean where species (aquaculture organisms like shrimp) perform at peak capacity. They analyze the water's makeup and culture single or mixed bacteria they find there to use as probiotics. Their culture does not involve any sort of chemicals or toxins. These are fortified with naturally occurring phytoplankton, amino acids, a wide range of vitamins and minerals, an important variety of anti-oxidants and proteolytic enzymes, as well as an array of nucleic acids. These are the paramount keepers of the code of life, which appears to be in charge of growth and continuous cell repair.

The hypothesis of stimulation of the immune system of aquatic organisms may be also considered. Many immunostimulants have been tested on fish and shellfish, and some of the originated from microbial cell walls, eg. glucans (Anderson, 1992; Huang,C.C., Song, Y.L., 1999; Song, Y.L., Huang, C.C., 2000; Bricknell, I., Dalmo, R.A., 2005). It is possible that autochthonous microbiota may stimulate the immune response of aquatic animals to enteric pathogens, as reported in shrimp (Gullian, M., et al, 2004) and in land animals (Gaskins, 1997).

Bioremediation – Concept:

The newest attempt being made to improve water quality in aquaculture is the application of probiotics and/or enzymes to the ponds. This type of biotechnology is known as *"bioremediation"*, which involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and get rid of undesirable waste compounds.

Probiotics and Enzymes

Probiotics

The concept of biological disease control, particularly using microbiological modulator for disease prevention, has received widespread attention. A bacterial supplement of a single or mixed culture of selected non-pathogenic bacterial strains was termed probiotics.

'Probiotics' the term was firstly coined by Parker (1974) and originated from two Greek words 'pro' and 'bios' which mean 'for life'. Organisms -- be they human, cattle, chicken, fish, prawn or shrimp -- require good bacteria to break down nutrients for digestion. Living systems require bacteria to decompose waste. "Probiotics" generally includes bacteria, cyanobacteria, fungi, etc. They may be called as "Normal micro biota" or "Effective micro biota". In literature, probiotic bacteria are generally called the bacteria, which can improve the water quality of aquaculture, and (or) inhibit the pathogens in water thereby increasing production. "Probiotics", "Probiont", "Probiotic bacteria", "Beneficial bacteria", or "Friendly bacteria" are the terms synonymously used for probiotic bacteria.

The theory of ecological prevention and cure in controlling the insect pest of terrestrial higher-grade animals and plants has been in practice for long time, and has achieved remarkable success. The bio-controlling theory has been applied to aquaculture and many researchers attempt to use some kind of probiotics in aquaculture water to regulate the micro flora of aquaculture water, control pathogenic microorganisms, to enhance decomposition of the undesirable organic substances in aquaculture water, and improve ecological environment of aquaculture. In addition, the use of probiotics can increase the population of food organisms, improve the nutrition level of aquacultural animals and improve immunity of cultured animals to pathogenic microorganisms.

According to some recent publications, in the aquaculture the mechanism of action of the probiotic bacteria have several aspects.

- probiotic bacteria competitively exclude the pathogenic bacteria or produce substances that inhibit the growth of the pathogenic bacteria (eg. Bacitracin and polymyxin produced by *Bacillus* sp.).
- provide essential nutrients to enhance the nutrition of the cultured animals.
- provide digestive enzymes to enhance the digestion of the cultured animals.
- probiotic bacteria directly uptake or decompose the organic matter or toxic material in the water improving the quality of the water.

Hence, the probiotics can decompose the excreta of fish or prawns, remaining food materials, remains of the plankton and other organic materials to CO_2, nitrate and phosphate. These inorganic salts provide the nutrition for the growth of micro algae, while the bacteria grow rapidly and become the dominant group in the water, inhibiting the growth of the pathogenic microorganisms.

The photosynthesis of the micro algae provide dissolved oxygen for oxidation and decomposition of the organic materials and for the respiration of the microbes and cultured animals. This kind of cycle improves the nutrient cycle, and it can create a balance between bacteria and micro algae, and maintaining a good water quality environment for the cultured animals.

Types of Probiotics

Non-viable probiotics	These are dead.
Freeze-dried probiotics	These will die rapidly upon leaving refrigeration.
Fermentation probiotics	These are produced through fermentation.
Viable probiotics	This is live with guaranteed shelf life, guaranteed number of organisms, have a protocol for counting and to be very stable and efficacious. (produce many benefits).

Probiotics and their Role:

Bacillus sp.	→	Mineralization and Breakage of proteins
Nitrosomonas sp.	→	Oxidation of ammonia
Nitrobacter sp.	→	Oxidation of nitrites
Aerobacter sp.	→	Reduction of organic matter
Cellulomonas sp.	→	Breakage of plant material

Beneficial effects of probiotics may be mediated by:

- Neutralization of toxin
- Suppression of viable count
- Production of antibacterial compounds
- Competition for adhesion sites
- Alternation of microbial metabolism
- Stimulation of immunity in the host
- Accelerate the sediment decomposition by producing organic acids
- Production of hydrogen peroxide
- Production of enzymes

Application of Probiotics in Aquaculture:

- To regulate the microflora of aquaculture water.
- To control pathogenic microorganisms.
- To enhance decomposition of the undesirable organic substances in aquaculture water and improve ecological environment of aquaculture by minimizing the toxic gases like ammonia, nitrite, hydrogen sulfide, methane etc.
- To increase the population of food organisms.
- Improves the nutrition level of aquaculture animals and improve immunity of cultured animals to pathogenic microorganisms.
- The frequent outbreaks of diseases can be prevented.

Enzymes

Enzymes are organic catalysts formed naturally in living cells. They are compounds, which accelerated the rate at which chemical reactions occur and they remain unchanged after the reaction is completed. Thousands of enzymes exist in nature and are responsible for life.

Enzymes work by breaking the chemical bonds that hold compounds together, releasing smaller, more readily absorbed compounds (Julio, H. Cordova-Murueta, et al., 2004). For example, proteases work on proteins, amylases work on starches, cellulases work on cellulose and lipases work on lipids or fats. Recall that cellulose is the major cell wall material in plants and is therefore quite durable.

Nitrogen Cycle

Ammonia is the principal excretory product of most aquatic organisms. Inputs of ammonia cannot be eliminated from the water body. But ammonia is toxic, acutely and chronically, to fish and invertebrates thus it is a critical water quality factor. Ammonia should be maintained below 0.1 mg/L (total ammonia). The most efficient way to do this is by the establishment of a biological filter. A biological filter is a collection of naturally occurring bacteria, which oxidize ammonia to nitrite, and other bacteria, which then convert nitrite to nitrate. Nitrite is formed either by the oxidation of ammonia (nitrification) or the reduction of nitrate (denitrification). Nitrite is toxic to fish and some invertebrates and should be maintained below 0.1 mg/L. It is also a critical water quality factor.

Nitrate is the end product of nitrification. The vast majority of aquaculture ponds accumulate nitrate as they do not contain a denitrifying filter. In general, nitrate should be maintained below 50 mg/L (measured as NO_3-N) but it is not a critical water quality factor.

The most common ways to reduce nitrate are water changes and growing live plants. More sophisticated systems such as denitrifying filters are also available. A denitrifying filter creates an anaerobic region where anaerobic bacteria can grow and reduce nitrate to nitrogen gas. But they can be complex and easily disturbed which kills the anaerobic bacteria. A poorly run denitrifying filter does not convert nitrate all the way to nitrogen gas but instead produces nitrite.

The nitrogen 'cycle' is the oxidation of ammonia to nitrite by bacteria of the genus *Nitrosomonas* and the subsequent oxidation of the nitrite to nitrate by bacteria belonging to the genus *Nitrobacter*. It is easiest to visualize the nitrogen cycle as an endless loop divided into four phases, as follows:

- Fish, prawn and shrimp excrete ammonia as waste from their gills, kidneys and normal respiration. Ammonia also develops from unconsumed feeds, shell moults of prawn and shrimp, dead algae, zooplankton etc by the microbial activity.

- A species of bacteria called *Nitrosomonas* converts this ammonia into nitrite

  $(2NH_4^+ + 3O_2 = 2NO_2^- + 4H^+ + 2H_2O)$.

- A second species of bacteria called *Nitrobacter* converts this nitrite into nitrate

  $(2NO_2^- + O_2 = 2NO_3^-)$.

- Algae and aquatic plants utilize nitrate to produce chlorophyll, which are in turn consumed by zooplankton and then by fish, prawn and shrimp. Thence the cycle repeats.

These bacteria are important to aqua farmers because without them it is difficult to maintain healthy environmental conditions in the aquaculture ponds.

The biochemical reaction of *Nitrosomonas* sp.: Energy generation in Nitrosomonas by two enzymes, Ammonia monooxygenase (AMO) and Hydroxylamine oxidoreductase (HAO) are involved in the oxidation of ammonia to nitrite.

$$NH_3 + O_2 + 2H^+ \xrightarrow{\textbf{AMO}} NH_2OH + H_2O$$

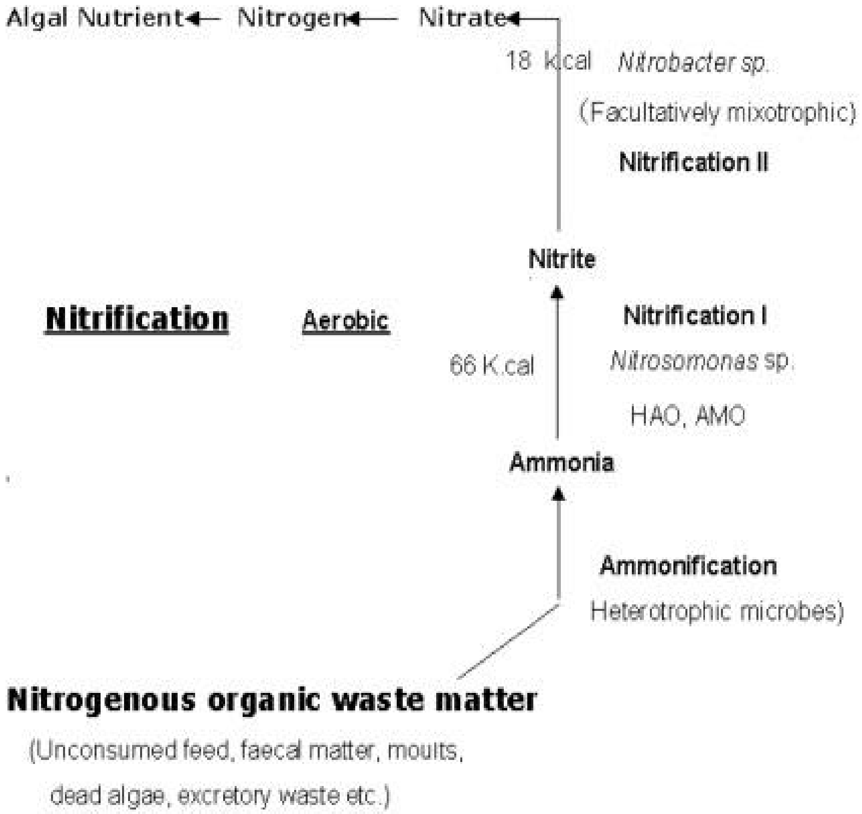
$$NH_2OH \xrightarrow{\textbf{HAO}} NO_2^-$$

The biochemical reaction of *Nitrobacter* is a very simple reaction, involving the cytochrome system as follows:

$$NO_2^- \longrightarrow NO_3^-$$

Nitrobacter sp. is facultatively mixotrophic and capable of growing anaerobically with nitrate as electron acceptor, producing nitrite, nitric oxide and nitrous oxide and then to nitrogen gas. Aerobically it oxidizes nitric oxide to nitrite and thence to nitrate. All tricarboxylic acid cycle enzymes are present (eg. Carboxysomes, poly-beta-hydroxybutyrate (PHB) and polyphosphate granules).

Fig. 1, Nitrification



The nitrifying bacteria are gram-negative, non acid-fast rods, which may be pleomorphic or coccoid (Nitrobacter). They may be motile. The main component of the gram-negative cell wall is lipopolysaccharide (LPS). Additionally there is present phospholipid, protein, lipoprotein and a small amount of peptidoglycan. Hence, the component of cell wall of most gram-negative bacteria is associated with endotoxic activity, with which are associated the pyrogenic effects of gram-negative infections like vibriosis. Sixty-six kilocalories of energy are liberated per gram atom of ammonia oxidized. Eighteen kcal of energy is liberated per gram atom of nitrite oxidized.

Nitrification is an obligate aerobic, oxidizing process. This means that it can only occur in an environment, which contains oxygen and the process produces electrons. In general **denitrification** is a process of bacteria converting nitrate to other substances. It is an anaerobic, reducing process. This means that it occurs in environments without oxygen and the process accepts electrons.

Denitrification is defined as the transformation of nitrate to dinitrogen. Dinitrogen is a gas that is harmless and will bubble out of the system. Between the starting product (nitrate) and the end product (dinitrogen) there are three intermediate products, these are in the order in which they are produced nitrite (NO_2-), Nitric Oxide (NO) and nitrous oxide (N_2O).

Hence, denitrification, like nitrification, is a multi-step process with many intermediate compounds produced before the final product is generated. In most cases these intermediate products are toxic like ammonia and nitrite. Thus denitrification does proceed to complete its process and to expel dinitrogen gas.

It is worth to mention that the nitrate, which is the end product of nitrification process is the major nutrient for the growth of primary producers i.e. phytoplankton as well as the microbes. Hence, the micro flora and fauna for their growth will utilize the nitrate and makes limit the process of denitrification.

Another difference between nitrification and denitrification is the type of bacteria, which perform the processes. Nitrification is done by what are called autotrophic bacteria. This term means the bacteria get the carbon they need for cell growth from carbon dioxide. Denitrifying bacteria are heterotrophic bacteria. This means they get their carbon from organic carbon sources such as methane, sucrose or glucose.

In addition these probiotics controls the formation of toxic hydrogen sulfide by eliminating the *Desulfovibrio desulfuricans*, the hydrogen sulfide producing bacteria, through antagonism. The probiotics converts the toxic sulfide to nutritive sulphate.

Conclusion:

The probiotics can decompose the excreta of shrimp, prawn or fish, remaining food materials, remains of the plankton and other organic materials to CO_2, nitrate and phosphate. These inorganic salts provide the nutrition for the growth of micro algae, while the bacteria grow rapidly and become the dominant group in the water, inhibiting the growth of the pathogenic microorganisms. The photosynthesis of the micro algae provide dissolved oxygen for oxidation and decomposition of the organic materials and for the respiration of the microbes and cultured animals. This kind of cycle may improve the nutrient cycle, and it can create a balance between bacteria and micro algae, and maintaining a good water quality environment for the cultured animals (Rao, A. V., 1999).

Probiotics reduce the level of infection and mortality in aquaculture organisms. Probiotics compete with the disease-causing bacteria for food. Customarily, ponds have indigenous bad bacteria that leisurely eat on an abundance of undigested food, faecal matter, and dead algae. When farmers suddenly introduce a large quantity of these good bacteria -- probiotics -- into a pond, the newcomers eliminate the pre-existing bad bacteria out of the nutrient queue. The old bacteria, often-bad bacteria, never having had to compete for food, cannot keep pace with the aggressive probiotics.

Probiotics excretions make the pond medium less inhabitable for bad bacteria. Not only do probiotics bacteria have terrific appetites; they excrete enzymes -- exoenzymes -- as a natural byproduct of their metabolic activity, just as human's sweat. The enzyme excretions infuse and spread throughout the pond medium, changing its chemistry, resulting destroy of bad bacteria. Hence, probiotics therapy greatly improves pond water quality and reduces the pollution level of effluent before its release into the environment.

Probiotics speed the breakdown of organic waste fragments (dissolved proteins and unused feed), thus lessening sludge build-up. If sludge is not removed or does not decompose, dangerous concentrations of sulfide, nitrite, ammonia and various organic acids can occur. While the exo-enzymes of good bacteria go to work on larger waste particles and breaking them down through chemical reaction. As a result, there are fewer harmful chemicals in the medium.

Probiotics balance algae growth through providing nutrients by their bio-degradation activity. Probiotics utilizes phosphate for their body metabolic activities and hence prevents over growth (heavy blooms) of algae by limiting the phosphate. Dead algae are one of bacteria's favorite foods. Also probiotics diminish nutrients normally consumed by algae and results less slime in the ponds.

Probiotics make better habitat for culture organisms. With less accumulation of organic matter on the pond bottom, more oxygen can penetrate the sediment. For eg. Prawn and shrimp characteristically burrow in the sediment. By loosening the sediment, probiotics make this burrowing easier. Moreover, they diminish the toxin level in the sediment itself.

Probiotics maximize fish, prawn and shrimp nutrition. In addition to consuming the feed administered by farmers, prawn and shrimp graze on tiny zooplankton that is present in the water supply. Zooplankton eats bacteria. Probiotics are another nutrient for existing zooplankton in the pond medium, thus invigorating the zooplankton population, and strengthen up the food supply to culture organisms.

Probiotics optimize the immune systems of culture organisms, increasing their resistance to disease. By upgrading the pond environment, augmenting the food supply, and making habitat more comfortable, probiotics fortify fish, prawn and shrimp immune system.

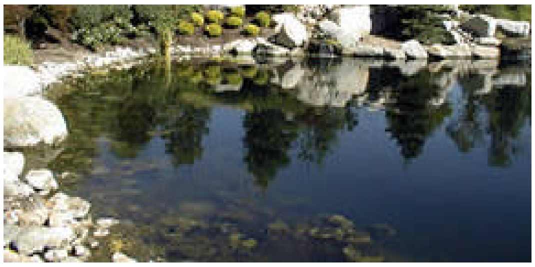
The application of probiotics in aquaculture shown promise, but further investigations may be expected with propagation of molecular approaches to analyse bacterial communities (Raskin et al., 1997; Wallner et al., 1997; Hugenholtz et al., 1998; Gatesoupe, F.J., 1999).

POND – BIOREMEDIATION

BEFORE

AFTER

BEFORE **AFTER**



References:

Anderson, D.P., 1992. Immunostimulants, adjuvants and vaccine carriers in fish: applications to aquaculture. Annu. Rev. Fish Dis. 2, 281-307.

Aubin, J., et al., 2005. Trial of probiotics to prevent the vertebral column compression syndrome in rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquaculture Research 36, 758.

Bricknell, I., Dalmo, R.A., 2005. The use of immunostimulants in fish larval aquaculture. Fish and Shellfish Immunology, 19, 457-472.

Direkbusarakom, S., Yoshimizu, M., Ezura, Y., Ruangpan, L., Danayadol, Y., 1998. *Vibrio* spp., the dominant flora in shrimp hatchery against some fish pathogenic viruses. J. Mar. Biotechnol. 6, 266-267.

Gaskins, H.R., 1997. Immunological aspects of host/microbiota interactions at the intestinal epithelium. In: Mackie, R.I., With, B.A., Isaacson, R.E. (Eds.), Gastrointestinal Microbiology, Vol. 2, Gastrointestinal Microbes and Host Interactions. Chapman & Hall Microbiology Series, International Thomson Publishing, New York, pp. 537-587.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture, Aquaculture 180, 147-165.

Gullian, M., Thompson, F., Rodriguez, J., 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in Penaeus vannamei. Aquaculture 233, 1-14.

Huang, C.C., Song, Y.L., 1999. Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (Penaeus monodon). Developmental and Comparative Immunology 23, 545-552.

Hugenholtz, P., Pitulle, C., Hershberger, K.L., Pace, N.R., 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. 180, 366-376.

Jordan Lin, C.T., et al., 2005. Awareness of foodborne pathogens among US consumers. Food Quality and Preference 16, 401-412.

Julio, H. Cordova-Murueta, et al., 2004. Effect of stressors on shrimp digestive enzymes from assays of feces: an alternate method of evaluation. Aquaculture 233, 439-449.

Kamei, Y., Yoshimizu, M., Ezura, Y., Kimura, T., 1987. Screening of bacteria with antiviral activity against infectious hematopoietic necrosis virus (HNV) from estuarine and marine environments. Bull. Jpn. Soc. Sci. Fish. 53, 2179-2185.

Kamei, Y., Yoshimizu, M., Ezura, Y., Kimura, T., 1988. Screening of bacteria with antiviral activity from fresh water salmonid hatcheries. Microbiol. Immunol. 32, 67-73.

Kozasa, M., 1986. Toyocerin (*Bacillus toyoi*) as growth promoter for animal feeding. Microbiol. Aliment. Nutr. 4, 121-135.

Maeda, M., Nogami, K., Kanematsu, M., Hirayama, K., 1997. The concept of biological control methods in aquaculture. Hydrobiologia 358, 285-290.

Moriarty, D.J.W., 1997. The role of microorganisms in aquaculture ponds. Aquaculture 151, 333-349.

Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351-358.

Parker, R.B., 1974. probiotics. The other half of the antibiotics story. Anim. Nutr. Health 29, 4-8.

Porubcan, R.S., 1991a. Reduction of ammonia nitrogen and nitrite in tanks of *Penaeus monodon* using floating biofilters containing processed diatomaceous earth media pre-inoculated with nitrifying bacteria. Program and Abstracts of the 22nd Annual Conference and Exposition, 16-20 June 1991, San Juan, Puerto Rico. World Aquaculture Society.

Porubcan, R.S., 1991b. Reduction in chemical oxygen demand and improvement in *Penaeus monodon* yield in ponds inoculated with aerobic Bacillus bacteria. Program and Abstracts of the 22nd Annual Conference and Exposition, 16-20 June 1991, San Juan, Puerto Rico. World Aquaculture Society.

Rao, A. V., 1999. Studies on some Ecological and Pathological aspects in shrimp farms of *Penaeus monodon* (Fabricius) at Vakapadu, Visakhapatnam District", Ph.D. Thesis. Andhra University, India.

Raskin, L., Capman, W.C., Sharp, R., Poulsen, L.K., Stahi, D.A., 1997. Molecular ecology of gastrointestinal ecosystems In: Mackie, R.I., With, B.A., Isaacson, R.E. (EDS.), Gastrointestinal Microbiology, Vol. 2, Gastrointestinal Microbes and Host Interactions. Chapman & Hall Microbiology Series, International Thomson Publishing, New York, pp. 243-298.

Song, Y.L., Huang, C.C., 2000. Application of Immunostimulants to Prevent Shrimp Diseases, Recent Advances in Marine biotechnology, Vol.5, Immunobiology and Pathology, 173-187.

Sugita, H., Hirose, Y., Matsuo, N., Deguchi, Y., 1998. Production of the antimicrobial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. Aquaculture 165, 269-280.

Timmermans, L.P.M., 1987. Early development and differentiation in fish. Sarsia 72, 331-339.

Vadstein, O., 1997. The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture 155, 401-417.

Villamil, L., Figueras, A., Planas, M., Novoa, B., 2003. Control of *Vibrio alginolyticus* in *Artemia* culture by treatment with bacterial probiotics. Aquaculture 219, 43-56.

Wallner, G., Fuchs, B., Spring, S., Beisker, W., Amman, R., 1997. Flow sorting of microorganisms for molecular analysis. Appl. Environ. Microbiol. 63, 4223-4231.