MARINE *BACILLUS* SPECIES AS BIOREMEDIATORS IN AQUACULTURE PONDS

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By

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CERTIFICATE

This is to certify that the thesis entitled "***Marine Bacillus species as bioremediators in aquaculture ponds***" submitted by **Mr. Sampath Naik, S.T.** I.D. No. **MFK 917** in partial fulfillment of the requirements for the award of **Master of Fisheries Science** in **Fisheries microbiology** of Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of research work carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship or other similar titles.

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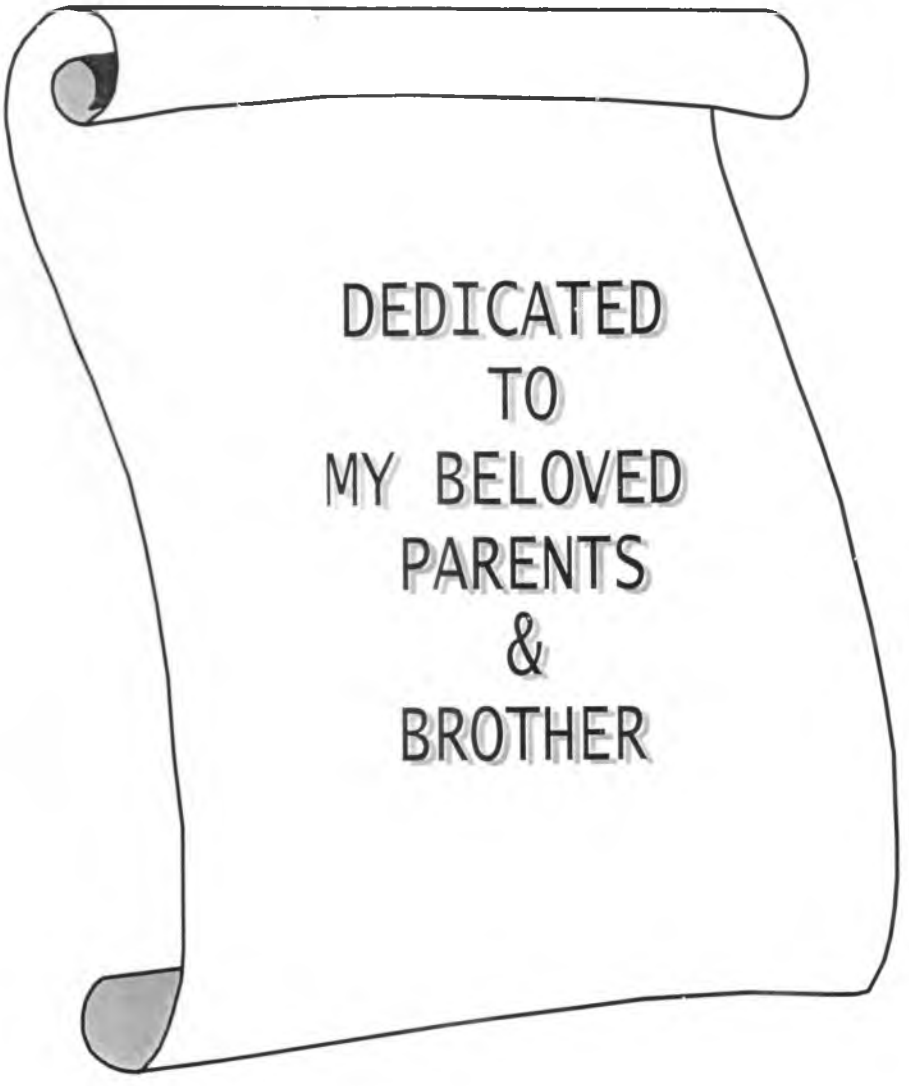
DEDICATED
TO
MY BELOVED
PARENTS
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Introduction

I. INTRODUCTION

Aquaculture which is concerned with the propagation and rearing of aquatic organisms under complete human control, involves the manipulation of at least one life stage of the cultured organism for increased production before harvest. Decline in fish catches from the marine environment in many parts of the world due to over-exploitation and pollution has led to looking of aquaculture to augment food production and this consequently contribute to economic development. Aquaculture was once considered an environmentally sound practice when traditional polyculture and integrated farming systems were practiced. In the present scenario, due to the avaricious nature of the farmers, there is deterioration of aquaculture environment.

Increased production is attempted by the expansion of land and water under culture and the use of sophisticated farming technologies for intensive culture that involve higher inputs such as water, feeds, fertilizers and chemicals. As a result, it has become a potential polluter of the aquatic environment and has caused of degradation of wetland areas (Pillay, 1992). With all the drawbacks, there is considerable progress in the past two decades in the sector resulting in significant quantities of fish food production, income and employment generation. By culturing several penaeids like *Penaeus monodon*, *Penaeus japonicus*, *Penaeus vannamei*, *Penaeus indicus,* fresh water prawn, *Macrobrachium rosenbergii*, and various other fish species, Asia has emerged as a major continent contributing to world aquaculture production. However the development of aquaculture has resulted in severe disease problems and changes in the quality of natural habitats through increased effluent discharges carrying

high levels of both organic and inorganic load. The wastes generated in hatcheries or aquaculture farms include residual food and faecal mater, metabolic by-products, residues of biocides and biostats, fertilizer derived wastes, wastes produced during moulting, collapsing algal blooms etc. (Sharma and Scheeno, 1999). These are responsible for deterioration of water quality as well as disease outbreaks. Therefore, for the sustainable development of aquaculture sector it is necessary to adopt eco-friendly aquaculture practices by minimizing impact on the environment.

To improve water quality in aquaculture, attempts have been made through the application of microbes and/or enzymes to the ponds. "Bioremediation", is a useful biotechnological approach which involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and to get rid of undesirable waste compounds. This is considered as an ecofriendly approach as it improves health of cultured animals and eliminates pathogens from the pond environment. Thus, a method to improve the ecological environment in an aquaculture setup has become the focus of attention of international aquaculture (Rao, 2008). Against this background, the present study was carried out with the following objectives:

1. To isolate *Bacillus* species from tropical environment
2. To study the bioremediation potential of the isolated species

Review of Literature

II. REVIEW OF LITERATURE

2.1. Bioremediation

2.1.1. Definition

Bioremediation can be defined as the addition of microorganisms or stimulation of indigenous microflora, in a polluted system with the aim of breaking down the pollutants to relatively acceptable substances (Edgehill, 1993). It is the technique of seeding purified bacteria or other microorganisms into the polluted system for reducing the impact of the contaminants (Ehrlich, *et al.*, 1988). These ecofriendly technologies do not pollute or cause adverse ecological consequences unlike other physical and chemical methods generally used, which during the process of cleaning up the pollutant wipe out a large numbers of beneficial microorganisms in the aquatic ecosystem that are essential for carrying out nutrient cycle and removal of toxic metabolites or contaminants (Austin, 1988).

The basic concepts of bioremediation are not new and these processes have been used in waste water and sewage treatments since many decades. The application of biological processes for disposal and control of waste from human activities is an established technology dating back to at least 4000 years (Barker, 1972). Microbiologists have been familiar with biodegradation (breaking down of organic matter) and bioconversion (chemical modification) for nearly a century (Skinner, 1990). In food industries, the microbial behaviour and activity have been best utilised for production of items such as beer, wine, yoghurt, cheese and sauerkraut. Similarly in chemical and pharmaceutical sector, microbes have been extensively used (Skinner, 1990).

The increase in the application of bioremediation as an environmental biotechnology has become possible because of the ability of existing microflora to metabolize contaminants and isolation and utilization of new microbes against different substrates. Further, microbial cell free enzymes, cell components and cell products are also being used as agents for bioremediation (Compeau *et al.*, 1991).

A paradigm shift is being seen from capture fisheries to culture fisheries since fish catches from the marine environment have been steadily declining in many parts of the world due to over-exploitation and pollution. Thus fisher folk are turning to aquaculture to augment food production and to contribute to economic development (Moriarty, 1998). Intensive and modern farming technologies that involve higher usage of inputs such as water, feeds, fertilizers and chemicals have led to the deterioration of water quality and disease outbreaks (Sharma and Scheeno, 1999). The physical, chemical and biological conditions of the culture environment have an influence on the health and productivity of aquatic animal. Exposure of aquatic animal to toxic chemicals like hydrogen sulphide, ammonia, and carbon dioxide leads to stress and ultimately disease (Ravichandran *et al.,* 2001). A successful bioremediation involves optimizing nitrification rates to keep low ammonia concentration, optimizing denitrification rates to eliminate excess nitrogen from ponds, sulphide oxidation to reduce accumulation of hydrogen sulphide, maximizing carbon mineralization to carbon dioxide to minimize sludge accumulation, maximizing primary productivity that stimulates shrimp production and also secondary crops, and maintaining a diverse and stable pond community where undesirable species do not become dominant (Bratvold *et al.,*1997).

Rao and Karunasagar (2000) demonstrated that lower accumulation of slime or organic matter in the pond bottom allows better penetration of oxygen into the sediment and provides a generally better environment for the farmed stock. The levels of luminous vibrio strains has been shown to decrease in ponds and tanks where selected probiotic strains of *Bacillus* species were added, leading to good survival rate (Moriarty *et al.,* 1999). Biodegradation of 2-naphthol and its metabolites, 1,2-naphthalene and 1,2-naphoquinone accumulated in wastewater was observed when a combination of fungus (*Aspergillus)* and bacterium (*Bacillus subtilis)* was used, thus lowering the accumulation of these two toxic metabolites (Zang *et al.*, 2010). The use of bioremediators in the culture of aquatic organisms is increasing with the demand for more environment friendly aquaculture practices (Gatesoupe, 1999; Gomez-Gil et *al.*, 2000; Verschure *et al.*, 2000; Irianto and Austin, 2002; Balaczar, 2003). No serious problems of water quality is observed during the initial stages of culture of aquatic organisms because of the small size of stocked animals, low metabolic rate and use of low amount of supplementary feed. However, as the animal grows, it results in rapid increase in biomass consequently resulting in water quality deterioration mainly due to the accumulation of metabolic waste, feed, and decay of biotic materials (Prabhu, *et al.*, 1999). Application of beneficial microorganisms such as *Lactobacillus, Bacillus, Nitrosomonas*, *Nitrobacter* and *Psuedomonas* has been found to be useful for controlling the pathogenic microorganisms and water quality (Prabhu *et al.*,1999; Shariff *et al.*, 2001; Irianto and Austin, 2002). *Bacillus* spp. have been evaluated as probiotics and are reported to improve water quality by influencing the composition of water borne microbial population and reducing the number of pathogens. Thus,

Bacillus is thought to antagonize potential pathogens in aquatic environment (Irianto and Austin, 2002).

The most beneficial microorganisms used as biological control agents in aquaculture belong to the lactic acid group (*Lactobacillus, Carnobacterium*), *Bacillus*, and *Pseudomonas* (Singh and Radhika, 2001). Application of beneficial bacterial isolates into shrimp culture system not only helps to suppress the pathogenic *vibrios* like *Vibrio harveyi* and *V. parahaemolyticus* but also reduces the opportunistic invasion of these pathogens in shrimps (Jameson, 2003). A good bioremediator is one which contains microbes that are effective in clearing aquaculture wastes from water. Members of genus *Bacillus* (*B. subtilis, B. cereus, B. licheniformis, B. coagulans)* and *Phenibacillus* (*Phenibacillus polymixa*,) are found to be effective for bioremediation of organic detritus. However, these bacteria are not normally present in required amount in the water column as their natural habitat is the sediment. They compete with the bacterial flora naturally present for the available organic matter, like leached or excess feed and shrimp faeces (Sharma, 1999). *Lactobacillus* is also used along with *Bacillus* to break down the organic detritus. These bacteria produce a variety of enzymes that break down proteins and starch to simple molecules (Haung, 2003). Ammonia is the principal excretory product of most aquatic organisms that gets into the water body. Due to its toxicity to fish and invertebrates, it is considered a critical water quality parameter. The principal source of ammonia to aquatic environment includes excreta from fish, sediment flux derived from the mineralization of organic matter and molecular diffusion from reduced sediment. However, cyanobacterial nitrogen fixation and atmospheric deposition become important in an aquaculture environment (Ayyappan and Mishra, 2003). Majority of aquaculture ponds accumulate nitrate as they do not contain a denitrifying filter. Denitrifying filters help to

convert nitrate to nitrogen and also favour anaerobic bacteria and reduction of nitrate to gaseous nitrogen (Rao, 2002).

The photosynthetic benthic bacteria that breakdown hydrogen sulphide in the pond bottom has been widely used in aquaculture to maintain a favorable environment (Singh and Radhika, 2001). These bacteria contain bacterio-chlorophyll that absorb light (blue to infrared spectrum, depending on type of bacterio-chlorophyll) and perform photosynthesis under anaerobic conditions (Haung, 2003). Photosynthetic bacteria of importance in aquaculture include members of *Chromatium, Thiosarcina, Thiospirillum, Thiocapsa, Thiiopedia, Chlorobium, Chloropsuedomoa*s and *Rhodospirillum* (Haung, 2003). The efficacy of Gram positive and Gram negative bacteria have also been tested for their bioremediation potential in shrimp culture by various researchers (Table 1). Presently different types of bioremediators are commercially available under different brand names (Table 2).

Table 1 : List of bacteria used as bioremediators in aquaculture (Source: Antony & Phillip, 2006).

Bioremediator	Source	Used on	Method of application
Gram positive bacteria			
Bacillus sp. 48	Common snook	*Centropomus undecimalis*	Added to water, reduced salinity
Bacillus sp	Commercial product	Penaeids	Water
Bacillus sp	Commercial product	Channel catfish	Spread in pond water
Mixed culture (mostly Bacillus sp)	Commercial product	*Brachionus plicatilis*	Mixed with water
Gram negative bacteria			
Aeromonas media	unknown	*Crassostrea gigas*	Mixed with water
Aeromonas CA2	unknown	*Crassostrea gigas*	Mixed with water
Photorhodobacterium	unknown	*Penaeus chenensis*	Mixed with water
Pseudomonas fluorescence	*Onchorhynchus mykiss*	*Onchorhynchus mykiss*	Mixed with water
Pseudomonas	*Onchorhynchus mykiss*	*Onchorhynchus mykiss*	Mixed in water

Table 2: Commercially available bioremediators for aquaculture applications (Antony & Phillip, 2006)

Sl.No.	Product	Microbial content	Company Firm
1	ABIL nitrifying package	Nitrifiers	Tropical Marine Centre,
2	Alken clear flo 1002	*Bacillus* sp.	Alken Murray Corp., .
3	Alken clear flo 1100	Nitrifying bacteria	Alken Murray Corp.,
4	Alken clear-flo 1400	3 species of *Bacillus* + 2 species of Nitrifying bacteria	Alken Murray Corp.,
5	Ammonix Prowins Bio	Nitrifying bacteria	Prowins Bio Tech Pvt. Ltd., .
6	Bactaclean	Nitrifiers	Enviro-Comp. Services, Inc., Dover,USA.
7	Biogreen	*Bacillus subtilis*	Activa Biogreen Inc., Wood .
8	Biostart	Bacillus sp	Bio-CAT. Inc., Virginia, USA.
9	BRF- 13A	*Nitrobacter, Nitrosomonas*	Enviro-reps., .
10	BRF-1A	Nitrifying bacteria	Enviro reps.,
11	BRF-4	*Nitrobacter, Nitrosomonas*	Enviro reps.,
12	BRF-4	Nitrifying bacteria	Enviro reps.,
13	BZT Aquaculture	Nitrifiers	United-Tech, Inc., , .
14	Detrodigest	*Bacillus sp.*	NCAAH, CUSAT, .
15	Eutroclear	Nitrifying bacteria	Bioremediate. Com, LLC, .
16	Nitroclear	*Nitrobacter, Nitrosomonas*	Bioremediate. Com, LLC, .

2.1.2. Bioremediation in waste water treatment

Carbonaceous and nitrogenous wastes in both organic and inorganic form are the chief components of sewage and waste which, upon disposal results in eutrophication of the sea and the fresh water ecosystem (Barker, 1972). Biological treatment or natural self purification of sewage involves mineralization of organic pollutants by a large variety of microorganisms. Many autotrophic and heterotrophic microbes are found to participate in mineralization of both organic carbon and organic nitrogen into the respective inorganic forms. Such beneficial microbial communities are utilized in treatment plants by inducing intense biological activity by developing a massive microbial culture that act on the organic waste (Kadota *et al.*, 1983). Primary sewage sludge and industrial effluents are the concentrated sources of highly degradable complex organic components such as polysaccharides, fats, proteins etc. The compounds like sugar, triglycerides, fatty acids, amino acids etc. are subjected to anaerobic fermentation leading to production of methane and other gases using methane oxidizing bacteria (Brock *et al.*, 1994). Removal of nitrogen through microbial denitrification and nitrification is a common practice followed for waste water treatment by seeding nitrifying bacteria to the treatment cells/bioreactors (Horsfall, 1979; Schuetzle *et al.*, 1982; Kadota, 1983).

2.1.3. Remediation of toxic chemical polluted environment

Microorganisms as major scavengers in nature are responsible for recycling most natural waste materials into harmless compounds. For the degradation of chemical industry wastes containing chlorinated chemicals (pesticides/ herbicides, industrial solvents, refrigerants etc.,), microorganism evolve new genes for encoding enzymes/ metabolites that help in their degradation (Parsek *et al.*, 1995).

Abiotic transformation involving processes like hydrolysis, oxidation-reduction reaction, photodegradation are the first step of degradation to yield products which are subsequently mineralized by soil/ water microorganisms. Conversely, the abiotic transformation can also complete the degradation following the microbial initiation (Hicky *et al.*, 1995). Herbicides such as acrylamides are partially degraded in soil by seeding selective group of bacteria which result in an intermediate product which subsequently get incorporated into humic materials.

2.1.4. Biotransformation of metals and metallic compounds

Environmental pollution of drinking water by toxic metals especially Pb, Hg, Cd etc. pose a potential hazard to the health and welfare of mankind. Many people in Japan have suffered from diseases like Ache-ache, Mina-mata, itai-itai etc. due to heavy metal pollution of coastal waters. Rapidly growing industrial operations usually release heavy metals, which even at low concentration can be biologically concentrated in natural food chain. In nature, microfloras are found to be involved in detoxification of metals involving oxidation/ reduction reactions (Shannon and Unterman, 1993). Thus, microorganisms offer a potential alternative to expensive conventional detoxification measures.

Chemolithotrophic microorganisms are generally involved in the oxido-reduction processes by using metals/ metal ion as electron acceptors/ receptors for their energy requirements (Austin, 1988; Rodina, 1972). Ishibashi, *et al.* (1990) reported remediation of toxic form of chromium ion (Cr+6), to less toxic form (QT3) by using *Pseudomonas putida*. Boseweker (1986) reviewed the potential of microbial metal recovery and detoxification from industrial wastes. They used sulphur oxidising bacteria, *Thiobacillus ferroxidans,* to detoxify metal sulphide contaminated waste water via oxidation reaction for transferring into water soluble

sulphates and allowed for bleaching. This bioleaching technology has proved to be useful for selectively removing metal containing pollutant complexes from bulky waste materials and subsequent disposal in non hazardous forms.

Using selenate respiring bacterium, *Thauera selenatis*, recovery of 91 to 96% of total Se in elemental form from agricultural drainage has been achieved (Cantafio *et al.*, 1996). A similar evaluation study of microbial leaching of Pb and Cu from highly contaminated aquatic sediment have shown positive results (Mercier *et al.*, 1996).

Some metals like As, Se and Hg become readily volatile when methylated or transformed to their metallic form (Shannon and Bartha, 1988). The bacterial strains exhibiting resistance towards increased metal concentration by adaptation and evolution of such genes are mainly exploited for environmental detoxification of several toxic metals.

2.1.5. Oil bioremediation

Natural biodegradation is found to be more effective in oil pollution. This involves seeding of natural/genetically engineered microorganisms having better hydrocarbon degradation capabilities than the flora existing at the contaminated site (Lee and Levy, 1989). This process of seeding with oil degrading bacteria was found successful during marine oil spill in Prince William Sound, Alaska in 1989 (Sheehy, 1993).

The observation that a large number of *Pseudomonas* species have the capability to degrade hydrocarbons encouraged extensive *in-situ* and field studies in oil bioremediation (Chakraborty, 1976; Williams, 1978; Marty, 1994). With the discovery that hydrocarbon degradation trait in those organisms were located in plasmids, genetically engineered strains containing several plasmids each carrying genes for the degradation of different classes of

petroleum hydrocarbons were developed (Friello *et al.*, 1976; Larsen, 1993). However, no single microbial species appears to possess the enzymatic capacity to metabolise more than two or three classes of compound present in petroleum hydrocarbon mixtures. Thus, different bacterial species in mixed culture may be required for degradation of a complex mixture such as crude oil (Westlake, 1982). Such a bacterial consortium with four hydrocarbonoclastic bacteria was found to degrade 70% of the crude oil where one member produced a biosurfactant, rhamnolipid which emulsified crude-oil efficiently for effective degradation by other members (Chhatre *et al.*, 1996). Similar effect had been reported earlier by Marty (1994) involving 4 hydrocarbonoclastic strains *in vitro* belonging to *Pseudomonas* and *Acinetobacter*. Slow release of oleophilic fertiliser (mostly nitrogenous) has been reported to increase rates of petroleum degradation which helped rapid proliferation of the seeded bacterial consortium (Marty, 1994; Atlas, 1995; Lacotte *et al.*, 1995).

2.2. Toxicity of ammonia, nitrite and sulphide to aquatic animals

The semi-intensive and intensive fish culture practices result in exponential increase of toxicants such as NH_3, NO_2, H_2S, CH_4 in grow out systems (Chen *et al.*, 1986, 1989). High concentrations of these toxicants often reduce growth of cultured animal and also in extreme conditions cause mortality either by directly affecting the organism or by precipitating latent microbial diseases.

2.2.1. Ammonia

Crustaceans being ammonotelic excrete ammonia as their chief metabolic/ excretory product which exists in aquatic environment in unionized (NH_3) and ionized form (NH_4) (Hanstein, 1970). Ammonia also gets into water through fertilization and microbial degradation of nitrogenous compounds of feed. Though ammonia is utilized by ammonia oxidising bacteria, it may also escape through other pathways like evaporation, physical agitation etc.

2.2.2. Nitrite

As an intermediate product in the biological oxidation of ammonia to nitrate, nitrite accumulation is a serious problem in intensive fish and shellfish culture (Alcaraz and Espina, 1995; Boyd, 1992; Chen and Lei, 1990). When absorbed by fish, NO_2 reacts with haemoglobin to form "methemoglobin" which impairs the oxygen carrying capacity and rate causing hypoxia and cyanosis leading to stress and consequent mortality which is known as "Brown blood syndrome" (Colt and Armstrong, 1981). The major source of "nitrite" for oxidation is presumably the ammonium oxidation in most of the habitats (Watson *et al.*, 1989). However, anaerobic nitrate reduction in microenvironments also adds up to the total NO_2-N level which would then diffuse into aerobic microenvironments to become available for nitrite oxidisers (Focht and Verstreate, 1977).

2.2.3 Hydrogen Sulphide

Reduced sulphur compounds accumulating in pond ecosystems cause great problems once anaerobic pockets develop in the sediment surface due to low dissolved oxygen content or poor water circulation (Boyd, 1990). Sulphide reacts with sediment metals to form a black precipitate which forms a black blanket on the sediment surface which often sloughs up and float on surface. This intensive blackening was reported to result in gill clogging and restrict free movement of bottom feeders thus affecting their growth and activity (Anon, 1993). High accumulation of sulphides directly affects the growth of aerobic miroflora. Sulphide toxicity level for fish was evaluated to be 0.01 to 0.05 ppm (Boyd, 1990).

2.3. Microbial processes involved in remediating water and sediment quality in aquaculture pond system

2.3.1. Bioremediation of organic matter

Organic compounds of all types are acted upon by various heterotrophic microorganisms which require organic matter as precursors for synthesizing cell substances and as source of energy for their growth and development. To achieve this, microorganisms first convert several complex substances into compounds of smaller energy reserve and later under aerobic conditions into original mineral substances for their usage (Campbell, 1977; Austin, 1988; Brock *et al.*, 1994). The release of CO_2 by microorganisms and low oxygen tension facilitates the growth of fermentative/ anaerobic degraders (Moriarty and Pullin, 1987). In the pond ecosystem, feed pellets, dead plankton, manure and metabolites constitute the principal organic matter reserve on which heterotrophs flourish. Thus providing aerobic environment

with sufficient population of heterotrophic degraders is advocated to hasten the process of decomposition in both semi intensive and intensive culture practices (Moriarty, 1996). Rate of degradation depends upon the composition of the organic matter and usually simpler forms like protein, sugar and fatty acids are acted upon initially followed by the degradation of complex forms like starch, fat, cellulose, chitin, lignin etc. This is also influenced by existing environmental conditions and under aerobic conditions the degradation process is observed to be faster due to proliferation of the microbial population involved (Rodina, 1972; Austin, 1988). The members of genus such as *Bacillus* and *Phenibacillus* are good examples of bacteria suitable for bioremediation of organic detritus. However, these are not normally present in required amounts in the water column as they are natural inhabitants of the sediment (Sharma, 1999). It has been thus suggested that members of genus *Bacillus* can be applied in the pond or bottom (Singh and Radhika, 2001) (as the part of bio-augmentation).

Table 3. Microbial genera involved in degradation of different carbon substrates (Agate and Panchanadikar, 1992)

Substrates	**Microorganisms**
Cellulose	*Bacillus* spp, *Clostridium* spp, *Pseudomonas* spp.
Hemicellulose	*Cytophaga* spp.
Lignin	*Flavobacterium* spp.
Starch	*Bacillus* spp, *Pseudomonas* spp, *Micrococcus* spp.
Pectin	*Xanthomonos* spp, *Corynebacterium* spp.
Chitin	*Vibrio* spp, *Pseudomonas* spp, *Bacillus* spp, *Aeromonas* spp.
Gelatin	*Pseudomonas* spp, *Bacillus* spp, *Vibrio* spp.

2.4. Nutrient cycle and microorganism in aquatic ecosystem

2.4.1. Bioremediation of nitrogenous compounds

Nitrogen is a major constituent of protein, the building block of all living microorganism. Microorganisms play an important role in cycling of nitrogenous substances through the process of nitrogen fixation, ammonification, and nitrification, denitrification. Ammonia is the principal excretory product of most aquatic organisms and is toxic to fish and invertebrates thus are a critical water quality factor. The principal sources of ammonia to aquatic environment are from fish excretion, sediment flux derived from the mineralization of organic matter, molecular diffusion from reduced sediment cyanobacterial nitrogen fixation and atmospheric deposition (Ayyappan and Mishra, 2003). Ammonia level of below 0.1 mg/L (total ammonia) is considered safe for aquatic organism. This can be achieved establishing a biological filter consisting of naturally occurring bacteria, which oxidize ammonia to nitrite, and 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. The vast majority of aquaculture ponds accumulate nitrate as they do not contain a denitrifying filter. In general, nitrate (NO_3-N) should be maintained below 50 mg/L but it is not a critical water quality factor. The nitrogen cycle (Fig 1.) involves 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. Nitrogen cycle can be considered as an endless loop consisting of four phases these are;

In aquaculture environment fish, prawn and shrimp excrete ammonia as waste from their gills, kidneys and normal respiration. Ammonia build up also takes place due to microbial activity on unconsumed feeds, shell moults of prawn and shrimp, dead algae, zooplankton etc. 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. Then the cycle repeats.

These bacteria are important to maintain healthy environmental conditions in the aquaculture ponds (Rao, 2008). Bacteriological nitrification is the most practical method for the removal of ammonia from closed aquaculture systems and it is commonly achieved by setting of sand and gravel bio-filter through which water is allowed to circulate. The common ammonia oxidisers include members of the genus, *Nitrosomonas, Nitrosovibrio*, *Nitrosococcus, Nitrolobus* and *Nitrospira*, and the nitrite oxidisers include *Nitrobacter, Nitrococcus* and *Nitrospira*. Besides, some heterotrophic nitrifiers also produce low levels of nitrite and nitrate and often use organic sources of nitrogen rather than ammonia or nitrite (Antony and Philip, 2006).

2.4.2. Nitrogen fixation

Nitrogen fixation in aquatic environment is carried out by prokaryotic microorganisms which fix free nitrogen to ammonia through the activity of nitrogenous enzymes and are refered to as diazotrophs. They include the bacteria and blue green algae. Dalton (1980) has recorded 69 species of bacteria and 18 species of blue green algae capable of fixing the nitrogen. Photosynthetic nitrogen fixing bacteria have been well documented in marine ecosystems (Sisler and Zobell, 1951; Pshenin, 1963; Truper and Genovase, 1968; Herbert, 1975, Lakshamanaperumalsamy and Chandramohan, 1975, and Mann and Steinke, 1989). Nitrogen fixation in anoxic sediments ascribed to bacteria is generally small in comparison to fixation by blue green algae (Brezonik and Harper, 1969; and Kevin and Brezonik, 1971). Nitrogen fixation associated with green algal communities in decomposing dead roots and leaves in mangroves has also been reported (Mann and Steinke, 1992).

2.4.3. Nitrogen mineralization (ammonification)

The degradation of organic nitrogen containing compounds and subsequent release of inorganic nitrogen as ammonia is refered to as ammonification. This process is driven by a wide variety of microorganisms. Under anoxic conditions, the released ammonia may either be exchanged with cations on soil particles or diffusion to be upper oxic layer where it is converted into nitrate (Fentchel and Blackburn, 1979). The ammonia released is assimilated by numerous plants and microorganisms (Gottschalk, 1979). The majority of the ammonium compounds released during ammonification are oxidized to nitrite or lost as gaseous ammonia.

2.4.4. Nitrification

In this process ammonia is first oxidized to nitrite and then to nitrate by different microorganisms. The formation of nitrate assumes greater importance in an ecosystem since it is either utilized by plants or lost by denitrification. The process of nitrification appears to be limited to a restricted number of autotrophic bacteria (Focht and Verstraete, 1977). The oxidation process are energy yielding and lead to assimilation of organic matter. Nitrification may also be carried out by heterotrophic bacteria and a few fungi (*Aspergillus sp.*) by utilizing organic carbon sources (Verstraete and Alexander, 1972). In water logged system, nitrification is limited by rates of diffusion of oxygen and ammonia. In organic rich soils such as mangroves oxygen availability is limited due to high heterotrophic activity (Vanderbrought and Billen , 1975).

2.4.5. Denitrification

Denitrification is a process by which bacteria convert nitrate to other substances which occur in environments to gaseous nitrogen. Hence denitrification, is a multi step process involves the production of intermediate compounds such as ammonia and nitrite. However, nitrate which is the end product of nitrification process is utilized by the primary producers such as phytoplankton and microbes thereby limit the denitrification process (Fig, 1).

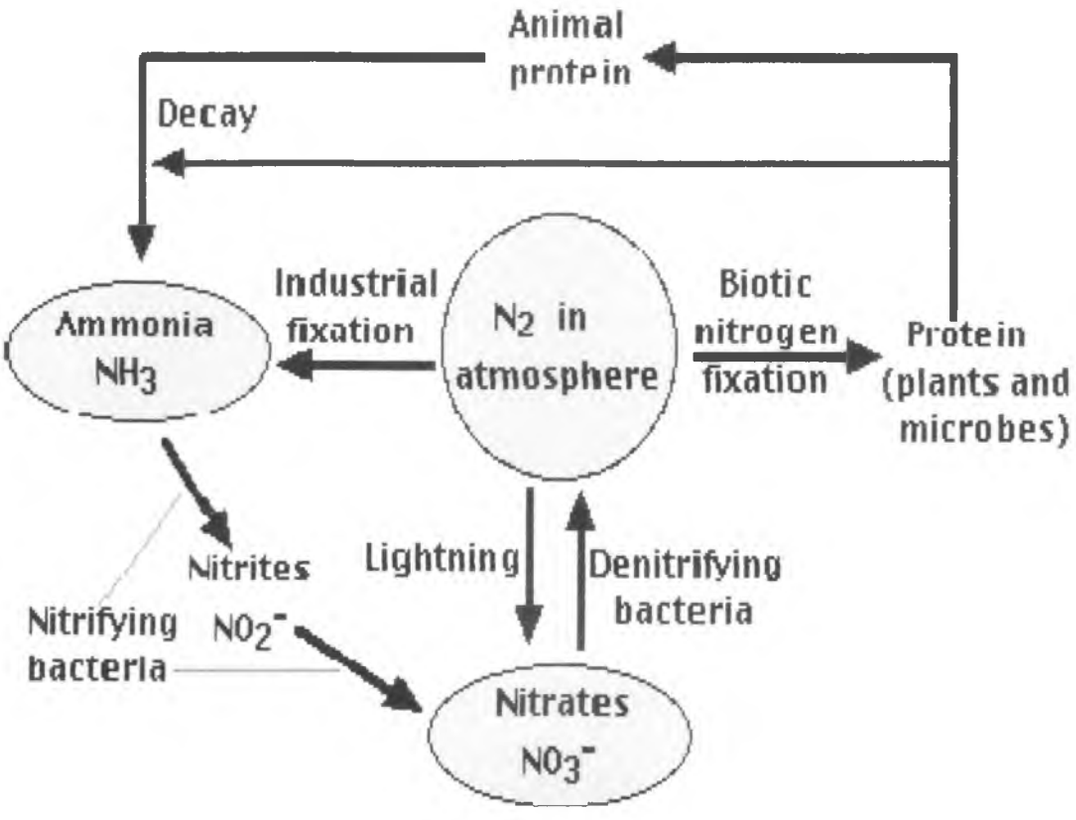


Fig.1.Nitrogen cycle

2.4.6. Bioremediation of sulphur compounds

Reduced sulphur compounds such as hydrogen sulphide accumulating in pond ecosystem cause great problems (Fig 2) once anaerobic pockets develop in the sediment surface due to low dissolved oxygen content or poor water circulation (Boyd, 1990). Sulphide reacts with sediment metals to form black precipitates forming a black blanket on the sediment surface which sloughs up and float on surface. Intensive blackening results in gill clogging and restricts free movement of bottom feeders, thus affecting their growth (Anon, 1993). High accumulation of sulphides directly affects the growth of aerobic micro flora. Unionised hydrogen sulphide is extremely toxic to fish at concentrations that may occur in natural waters and aquaculture operations (Bonn and Follis, 1967). Sulphide toxicity level for fish has been evaluated to be 0.01 to 0.05 ppm (Boyd, 1979, 1990). The photosynthetic benthic bacteria such as purple and green sulphur bacteria that break H_2S at pond bottom have been widely used in aquaculture to maintain a favorable environment (Singh and Radhika, 2001). These bacteria contain bacterio-chlorophyll that absorb light (blue to infrared spectrum, depending on type of bacterio-chlorophyll) and perform photosynthesis under anaerobic conditions prevailing in sediment water interface (Haung, 2003). Photosynthetic purple non-sulphur bacteria can decompose organic matter, H_2S, NO_2 and harmful wastes of ponds. The green and purple sulphur bacteria split H_2S to utilize the wavelength of light not absorbed by the overlying phytoplankton. The purple and green sulphur bacteria obtain reducing electrons from H_2S at a lower energy cost than H_2O splitting photoautotrophs and thus require lower light intensities for carrying out photosynthesis (Antony and Philip, 2006).

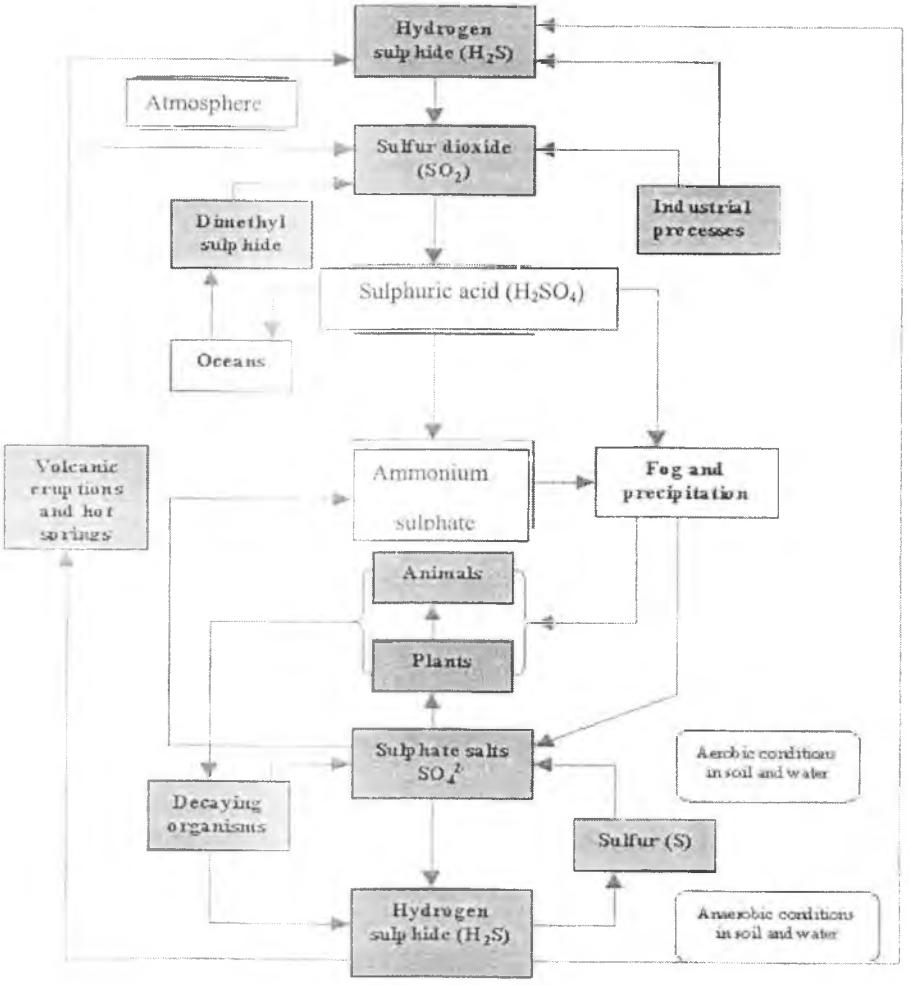


Fig 2. Sulphur cycle.

2.4.7. Phosphorus cycle

Phosphate is one of the most important limiting factor for plant life in many waters. Phosphorus is a vital element for all organisms and forms of nucleic acids, phospholipids, phophorylated sugars, phytin, adenosine triphosphate etc. Phosphorus cycle involves conversion of inorganic phosphorus to organic form and vice versa (Fig.3). Phosphorus is taken up by plants as pyrophosphates that are converted into organic phosphorus compounds from which phosphates are released mainly due to the action of microorganisms (Rheinheimer, 1992). During cycling, phosphorus may get immobilized due to adhesion to clay particles or formation of ferric or aluminium phosphate.

Solubilization of inorganic phosphate is carried out by a wide range of microorganisms such as *Pseudomonas, Serratia, Flavobacterium, Proteus, Arthrobacter, Streptomyces*, *Aspergillus and Rhizopus* (Boto, 1988). This solubilized phosphorus is taken up by phytoplankton and plants for production of organic substances. Although many organisms have the ability to hydrolyse phytate *in vitro*, this form of organic phosphate has a very strong affinity for adsorption on clay particles which prevent access by the phytases produced by the organisms.

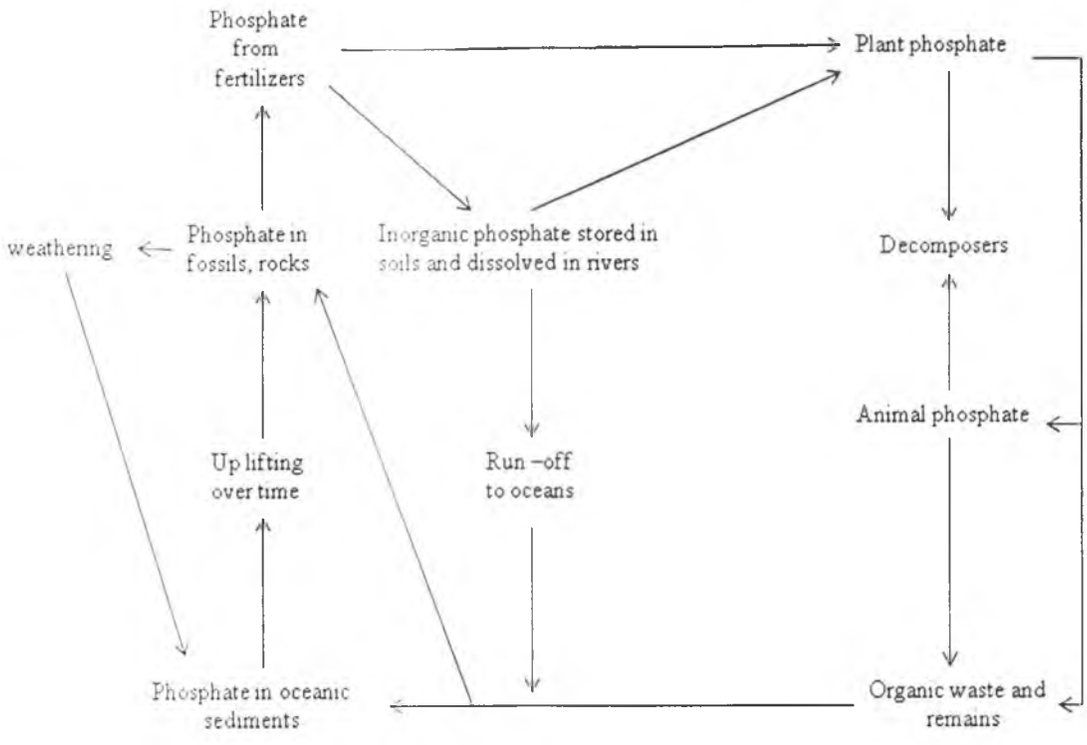


Fig 3. Phosphorus cycle.

2.5. Bioremediation in aquaculture

Aquaculture activity results in an exponential increase of toxic substances like NH_3, NO_2, BOD, and solids etc. which in turn result in the deterioration of water and soil quality (Ehrlich *et al.*, 1988; Boyd, 1990; Moriarty, 1986). Microorganisms play an important role in self purification of soil and water and as decomposers in any ecosystems (Fentchel and Blackburn, 1979) including pond ecosystem. The microbial food web forms an integral part of all aquaculture ponds having a direct impact on productivity and nutrient cycling. The control quality of rearing water in hatcheries involves adaptation of several strategies including use of several chemicals like ozone, chlorine, oxidising agents, chelating agents, and physical methods like filtration, aeration, heat etc. which have both beneficial and negative impacts (Dupree, 1981). The look out for harmless, inexpensive and easy to use technique has led to the application of bioremediation and bioaugmentation technology. However, use of bacterial products in aquaculture has both successful and unsuccessful results.

Earliest report on bioremediation was in 1981 by Bower and Turner who recorded accelerated nitrification in new sea water culture systems by seeding effective beneficial microorganisms from established system. Efficacy of a commercial bacterial suspension (Aqua Bacta Aid) containing *Nitrobacter* (NB), *Nitrosomanas* (NS), *Pseudomonas* and *Cellulomonas* has been tested by Boyd *et al,* (1984) to improve the water quality of a channel catfish pond. However, they did not have significant effect on the water quality parameters like NH_3-N, NO_2 -N, NO_3-N, Total P, Chlorophyll, COD, BOD etc. Similar evaluation study of the effectiveness of a common commercial water conditioner containing *Nitrosomonas*

(NS) and *Nitrobacter* (NB) for activating the biofilters for hatchery recirculatory system was reported by Labomascus *et al.,* (1987). They found significantly higher nitrification rate in seeded filters, both in fresh and marine water systems.

King (1986) compared the effect of a biofilter containing chemolithoautotrophic bacteria with various other filtration techniques for recycling effluent water for brackish water aquaculture. He observed biofilters to be effective for removal of NH_3 through nitrification. Such positive results of some bacterial consortia have been reported by Ehrlich *et al.*, (1988) who demonstrated its ability to accelerate nitrification, to decompose organic solids (10-12 cm/ microorganism) rapidly, reduce expensive algal growth (by over 80%) to facilitate oxygenation and to aid in transformation of aquaculture wastes into faunal biomass. The removal of organic deposits has helped to reduce the impact of aquaculture effluents on eutrophication of natural water body to which effluent was disposed off. Tidwell *et al.* (1992) showed the effect of plant extract from *Yucca shidigera* in reducing the concentration of NH_3 and NO_2 after 24 of application in channel catfish ponds. Chiayvaressajja and Boyd (1993) reported the failure of a bacterial product (ACCELOBAC) in reducing the total ammonia nitrogen concentration. On similar lines, Fungesmith and Hawthurn (1996) tested the efficacy of five commercially available bioremedial products under laboratory conditions and found none of them to have any effect on total NH_3 and NO_2 concentration.

In contrast to the use of live bacterial cells for seeding, Perfettini and Bianchi (1990) used an inocula of frozen cells of nitrifying bacteria for accelerating the conditioning of closed sea water system and found a 50% shortening in the time for establishing nitrification when enriched with bacterial inocula in comparison to control. Similar results of reduction in

ammonia and nitrite by seeded nitrifying bacteria in shrimp larviculture systems were observed by Chen et al. (1991).

In Thailand, reports of good production from intensive shrimp culture ponds (~6-9 ton/ha) by using commercial bacterial products for maintaining good water quality throughout the culture period indicates the positive effects of biremediators in field conditions (Anon, 1993, 1995). Similarly in Indonesia, a commercial product "EPICIN" is in good demand among farmers since they have obtained increased production through improved water quality comparison to untreated ponds (Anon, 1995). Krom *et al.*, (1995) suggested microorganisms such as *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Cellulomonas*, *Rhodopseudomonas*, *Nitrosomonas*, *Nitrobacter* etc. with bioremedial potential for use in pond aquaculture systems.

The need for application of useful microorganism to aquaculture environment is to increase their number. There are several reports of significant fluctuation in bacterial populations qualitatively and quantitatively both spatially and temporarily in different aquatic ecosystems like lakes (Chrost et al., 1986) rivers, freshwater ponds (Jana and Roy, 1985), brackish water environment (Rheinheimer, 1980) and shrimp culture pond (Ruangpan *et al.*, 1995; Moriarty, 1996). The bacterial dynamics certainly reflects fluctuation in their availability and enzymatic activity. Inhibition of bacterial activity due to residual antibiotics, pesticides, other chemicals and low dissolved oxygen may also determine the level of bacteria in any environment. Thus, there is need for addition of bacterial mixture or manipulation of the microflora of the deteriorated environment to hasten the process of mineralisation, thereby bringing about rapid purification (Ehrlich *et al*, 1988; Rao, 1997). According to Moriarty (1996), some bacteria

require certain specific conditions for their growth like light, dissolved oxygen, nutrients etc. Autotrophic nitrifiers need CO_2 as carbon source, ammonia for energy and grow better in the absence of light. After augmentation, bacteria may take time to build up in enough numbers to have a measurable effect on the accumulated organic matter. Therefore, the best alternative to achieve good results countering the unfavorable environmental factors is to supplement sufficient numbers of beneficial bacteria (Moriarty, 1996).

There are many microorganisms that are relatively resistant to conditions of unusually low/ high pH, DO and temperature, presence of heavy metals, (Hg, Pb, Cr, As), conditions normally prohibitive to life and these should be considered as potential bioremediators. Combination of genetic engineering techniques to introduce desired degradative phenotypes to host strains possessing tolerance/resistance to adverse environmental condition will make very powerful bioremediators. This approach may find increasing application in future as the practicality and desirability of this approach to treat hazardous wastes becomes widely accepted. In closed water aquaculture ecosystem, the use of genetically engineered microorganisms does not pose problem as it can be controlled (Maeda, 1994; Colwell, 1993; Matsunaga, 1995).

Materials and Methods

III. MATERIALS AND METHODS

3.1. Sample collection

To isolate the bacteria with bioremediation potential, water and sediment samples were collected from mangroves and shrimp farms situated close to Fisheries College, Mangalore. Water and sediment samples were collected at fortnightly intervals aseptically in sterile bottles and sterile plastic bags, respectively and brought to the laboratory for analysis.

3.2. Bacteriological analysis

3.2.1. Isolation of *Bacillus* species from the marine environment

Samples were suitably diluted using 0.85% physiological saline. 0.1ml of each dilution was spread in duplicate on to Tryptone soy agar containing 1% NaCl (TSAS) plates and incubated for 24 hrs at ambient temperature (29°±1° C). The representative colonies were picked up and purified on TSAS plates for further biochemical identification. Bacterial identification was made following the scheme suggested for Gram positive bacteria by Le-Chevalier *et al.*, (1980).

Media and reagents used in the study:

Reagents:

Gram staining reagents

Crystal violet (Huckers)

Solution A

Crystal violet 85% dye	2.0 g
Ethanol (95%)	20.0 ml

Solution B

Ammonium oxalate-monohydrate	0.2 g
Distilled water	20.0 ml

Solution A was diluted ten fold and mixed with an equal volume of solution B, filtered and stored.

Lugol's iodine

Iodine crystals	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml

Iodine crystals and potassium iodide were mixed and dissolved in distilled water, filtered and stored in dark bottles.

Safranin (Counterstain)

Safranine	2.5 g
Ethanol (95%)	100 ml

Safranin was added to 100 ml of alcohol (stock solution). To get the working solution, 10 ml of the alcoholic solution was diluted with 90 ml of distilled water.

Kovac's reagent

p-Dimethyl amino benzaldehyde (DMAB)	5.0 g
Amyl alcohol	75 ml
Conc. HCl	25 ml

P-DMAB was dissolved in amyl alcohol and then hydrochloric acid was added slowly. The reagent was stored at 4°C in dark bottles.

Methyl red indicator

Methyl red	0.1 g
Alcohol (95%)	300 ml

Methyl red was dissolved in ethyl alcohol and the volume made up to 500 ml, filtered and stored.

Oxidase reagent

1.0 g of the oxidase reagent (N,N,N,N,-tetramethyl phenylene diamine hydrochloride, Sigma company , USA) was added to 100 ml of distilled water. Whatman no.1 filter paper was cut and strips dipped in the reagent, dried and stored in dark at 4°C.

Nitrate reagent

Solution A:

Sulfanilic acid	8.0 g
5 N acetic acid	1.0 l

Solution B:

α- napthol	5.0 ml
5 N acetic acid	1.0 l

Physiological saline

Sodium chloride	8.5 g
Distilled water	1000 ml

The medium was autoclaved at 121°C for 15 minutes.

Tryptone soy Agar with 1% NaCl (TSAS)

Tryptone	10 g
Sodium chloride	10 g
Yeast extract	5.0 g
Agar	15 g
Distilled water	1000 ml
Final pH	7.3 ± 0.2

The medium was autoclaved at 121°C for 15 minutes, 20 ml portions were dispensed into sterile Petriplates.

Tryptone broth

Tryptone	10.0 g
Sodium chloride (NaCl)	5 g
Distilled water	1000 ml
pH	7.2 ± 0.2

The ingredients were dissolved in distilled water and sterilized by autoclaving at 121°C for 15 minutes.

T_1N_1 medium

Tryptone	10.0 g
Sodium chloride	5 g
Agar	20 g
Distilled water	1000 ml

The medium was heated to dissolve the ingredients, dispensed into tubes (3 ml) and autoclaved at 121°C for 15 minutes.

Indole broth

Tryptone	1.0 g
Sodium chloride	1.0 g
Distilled water	100 ml

The medium was dispensed into tubes (3 ml) and autoclaved at 121°C for 15 minutes.

Sugar fermentation test

Peptone	1.0 g
Sodium chloride	1.0 g
Sugar	1.0 g
Bromocresol purple	0.0016 g
Distilled water	1000 ml
Final pH	7.1 ± 0.2

The medium was heated to dissolve the ingredients, dispensed into tubes (3 ml) and autoclaved at 110°C for 10 minutes.

Simmon's citrate agar

Sodium citrate hydrate	2.0 g
Sodium chloride	5.0 g
K_2HPO_4	1.0 g
$NH_4H_2PO_4$	1.0 g
$MgSO_4$	0.2 g
Bromothymol blue	0.08 g
Agar	15 g
Distilled water	1000 ml
Final pH	6.9 ± 0.2

The medium was heated gently with occasional agitation, boiled for 2 minute until agar dissolved, dispensed into tubes (3-4 ml) and autoclaved at 110°C for 10 minutes. The medium was prepared as slants.

Starch agar

Peptone	5.0 g
Beef extract	3.0 g
Sodium chloride	10.0 g
Soluble starch	20. g
Agar	15.0 g
Distilled water	1000 ml
Final pH	7.2 ± 0.2

All the ingredients except starch were added to distilled water and heated. Soluble starch added slowly and boiled for few minutes until the dissolution was complete. The medium was cooled for 15 minutes and pH adjusted before autoclaving at 110°C for 15 minutes. The medium was poured to sterile petriplates.

Gelatin Agar

Peptone	5.0 g
Gelatin	20.0 g
Beef extract	3.0 g
Nacl	5.0 g
Distilled water	1000 ml
Final pH	7.2

All the ingredients were added to distilled water and heated for few minutes until the dissolution was complete. The medium was cooled at 15 minutes and pH adjusted before autoclaving at 110°C for 15 minutes. The medium was then poured to sterile petriplates.

Table 4: Artificial sea water (Lyman and Fleming, 1940)

Table A: Gravimetric salts

Salt	Molecular weight	g/kg^{-1} solution
NaCl	58.44	23.926
Na_2SO_4	142.04	4.008
KCl	74.56	0.667
$NaHCO_3$	84.50	0.196
KBr	119.01	0.098
H_3BO_4	61.83	0.026
NaF	41.99	0.003

Table B: Volumetric salts

Salt	Molecular weight	Mol/kg^{-1} solution
$MgCl_2.6H_2O$	203.33	0.053
$CaCl_2.2H_2O$	147.03	0.01033
Sr.Cl2.$6H_2O$	266.64	0.00009

Gravimetric salts and volumetric salts were mixed in 500 ml of distilled water, made up to 1 liter volume and then autoclaved.

Table 5: Biochemical characteristics of *Bacillus* spp. (Source: FDA, BAM, 2001)

Characteristics	As per the FDA bacteriological manual
Gram staining	+
Catalase	+
Motility	+
Nitrate reduction	+
Gelatin digestion	+
Anaerobic utilization of glucose	+
VP test	+
Starch hydrolysis test	+

3.3. Biochemical test:

A battery of biochemical tests (MacFaddin, 1980) were performed to identify the *Bacillus* sp.

3.3.1. Gram staining

To observe Gram reaction, young colony of cultures on TSAS was picked up and smeared on a slide, heat fixed and stained with crystal violet. After one minute treatment with mordant, it was decolorized with alcohol and counterstained for one minute with safranin, blotted dry and observed under the oil immersion objective by placing a drop of oil on the smear. Gram positive bacteria appeared purple and Gram negative bacteria pink in color.

3.3.2. Catalase test

Using an inoculating loop, transfer a small amount of the colony to a clean, dry glass slide. Immediately place one drop 3% hydrogen peroxide on the colony on the slide. Observe the slide for immediate formation of bubbles indicating the positive reaction.

3.3.3. Cytochrome oxidase test

Colony on TSAS was picked up and smeared on to moistened filter paper strips containing the oxidase reagent using a glass rod. Positive test was indicated by a dark purple coloration within 10 sec.

3.3.4. Motility test

A 6-8 h broth culture was suspended in a cavity slide and observed for motility under microscope.

3.3.5. Oxidative fermentation test (O/F test)

Ability of the cultures to ferment glucose oxidatively, fermentatively or both was tested on Hugh and Leifson's O/F medium.

Test cultures were stabbed simultaneously into the butt of two tubes. One tube was overlayed with sterile liquid paraffin (sterilized at 180°C for 1 h). Tubes were incubated at 37°C. Positive reaction was indicated by a change in color from purple to yellow in either of the tubes. The tubes were observed for a week and the reaction recorded as +/-, -/+, +/+ or -/- for O/F reaction.

3.3.6. Indole test

A loopful of test culture was inoculated into tryptone broth and incubated for 24 hrs at 30°C. Formation of a red ring upon the addition of 0.5 ml Kovac's reagent was considered a positive reaction. Since *Bacillus* is negative for indole (lack of deep red color at the surface of broth) all the cultures positive for indole production were discarded.

3.3.7. Simmons citrate test

Test cultures were inoculated on citrate agar slants by stabbing the butt and streaking the slant and tubes incubated at 30°C for 24 hrs. Presence of growth usually accompanied by color change from green to Prussian blue was considered as positive reaction.

3.3.8. Starch hydrolysis test

The water and sediment samples collected aseptically in sterilized bottles were suitably diluted with 0.85% physiological saline. Spread plate technique was followed by plating 0.1ml of each dilution on to the agar plates which were incubated at 30°C for 18 hrs.

The starch hydrolytic ability of the organism was tested by flooding the plates with iodine solution. Positive reaction was indicated by a clear zone surrounding the colonies (Plate 4).

3.3.9. Gelatin hydrolysis test

Protein decomposing ability of bacteria in water and sediment was tested by their ability to digest gelatin, a relatively resistant protein. The total number of gelatin hydrolyzing bacteria was determined by spread plate technique by spreading 0.1ml of each dilution on gelatin agar plates. Incubation was at room temperature (30±1° C) for 2-3 days. On appearance of well developed colonies, the plates were flooded with acidified mercuric chloride solution and the number of colonies surrounded by clear zones was considered as positive reaction.

3.3.10. Nitrate reduction test

To 3ml of an 18-24 hrs culture in nitrate broth, 2 drops of solution A and 2 drops of solution B of nitrate reagent were added. Appearance of orange coloration indicated positive reaction.

3.4. Experimental set up of microcosm for preliminary selection of isolates with potential for clean up

Eight glass beakers were cleaned and filled with 1cm thick sediment collected from the mangrove area and overlaid with 500 ml sterile sea water. Out of 8 beakers 2 served as control. The first control beaker (Beaker 1) was without addition of any feed, while to the second control beaker (Beaker 2) 2 g of feed was added. The selected bacterial isolates to be tested for their efficacy in clearing the blackening of soil bottom were grown individually in 10 ml test tubes containing 5ml TSB. After 2-3 days of incubation, to the treatment beakers 1, 2 and 3 bacterial cells grown in TSB were added. Clearing of blackening of soil bottom in each beaker was compared visually with that in control (Fig 4).

3.5. Testing of isolates for their ability to reduce ammonia levels and improving water quality parameters in microcosm.

Microcosms which simulate the environmental conditions prevalent at the site of aquaculture activity were set up in order to carry out the studies on effect of bioremediators.

3.5.1. Preparation of microcosm:

Eight plastic tubs of 7 liter capacity were cleaned, sun dried and filled with 1cm thick sediment collected from the mangrove area and overlaid with 7 lit of sea water. The sediment surface was leveled manually and the sea water added was allowed to settle for one day (Plate1).

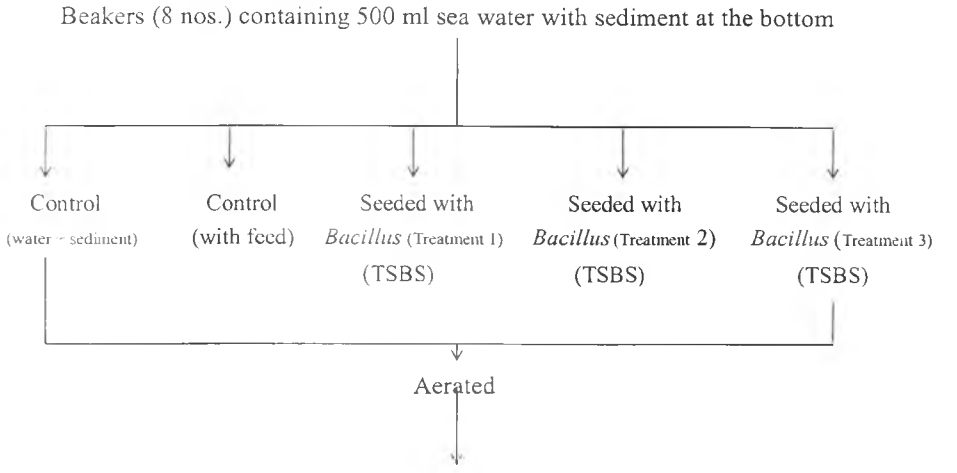


Fig 4: Flow chart of the preliminary microcosm experimental set up

Treatment 1: *Bacillus* sp grown in TSBS (trypticase soya broth)

Treatment 2: *Bacillus* sp grown in TSBS (trypticase soya broth)

Treatment 3: *Bacillus* sp grown in TSBS (trypticase soya broth)

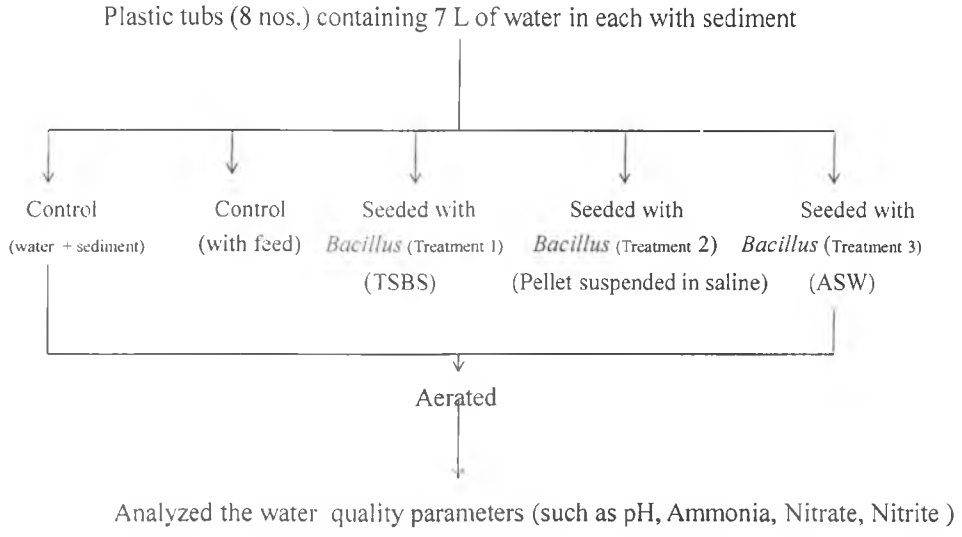


Fig 5: Flow chart of the microcosm experimental set up using *Bacillus* isolate 1 (Expt. 1)

Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Micr ocosm setup

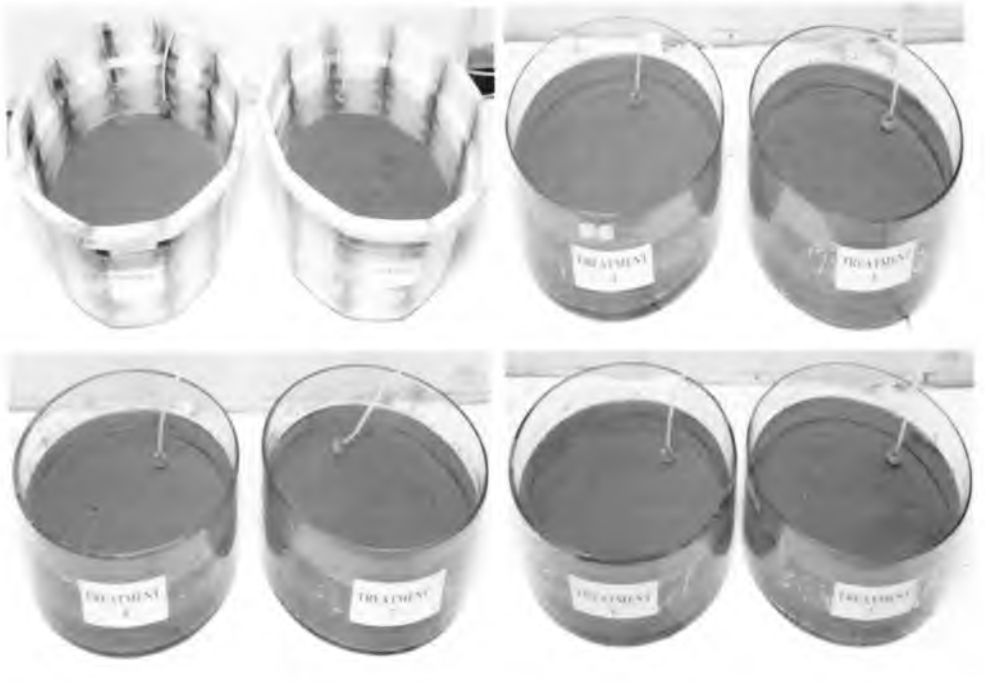


Plate 1: Microcosm experimental set up before addition of fish feed

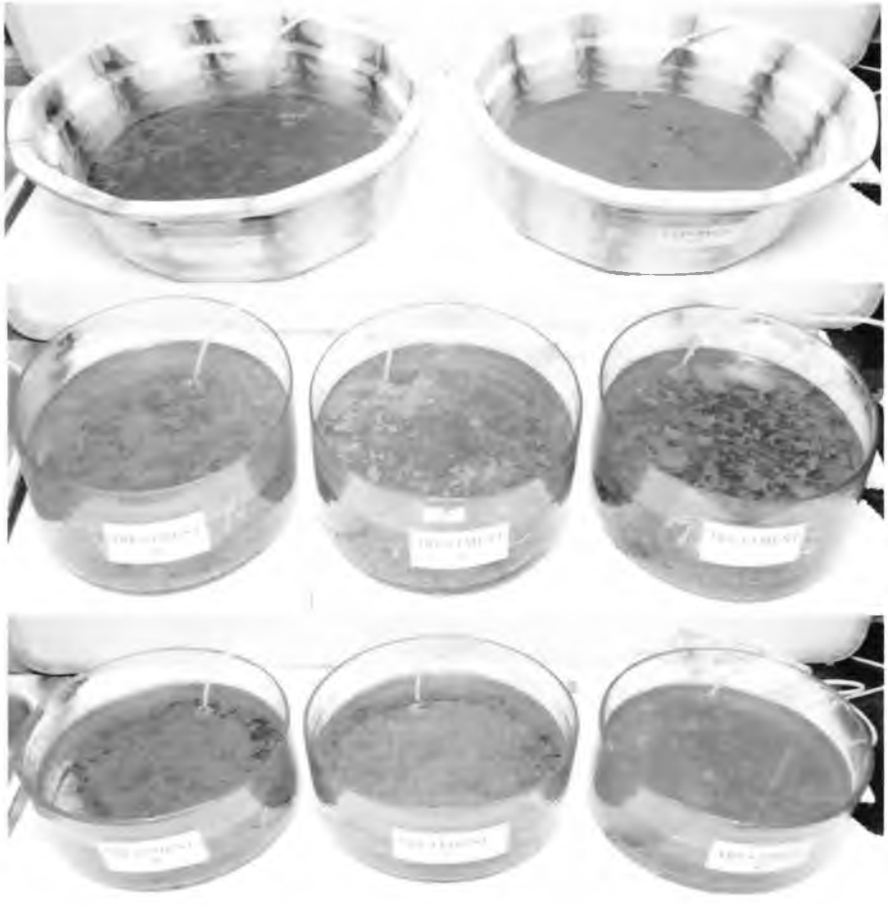


Plate 2: Microcosm experiment showing blackening in the bottom soil layer after addition of feed

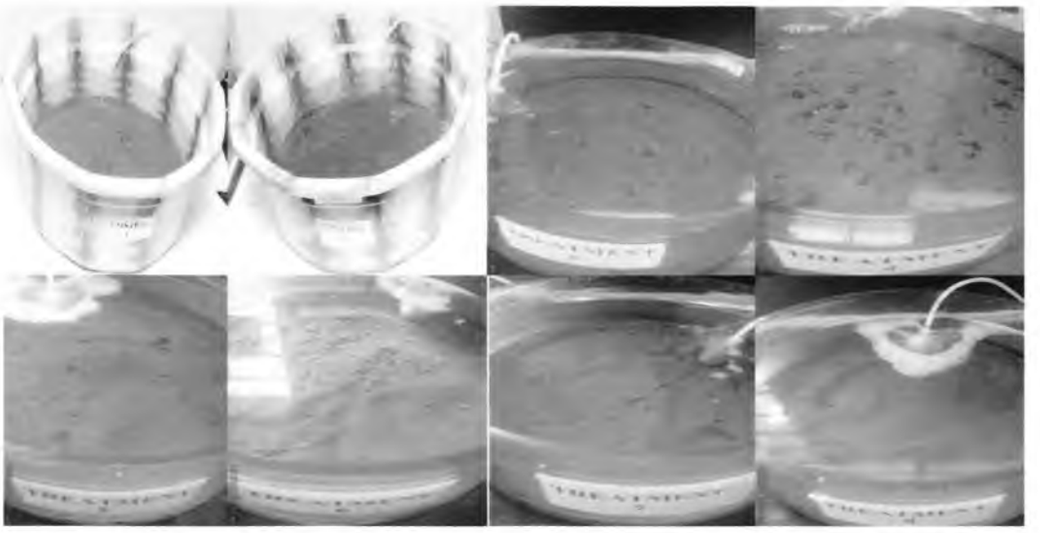


Plate 3: Effect of *Bacillus* sp in clearing blackening of soil bottom

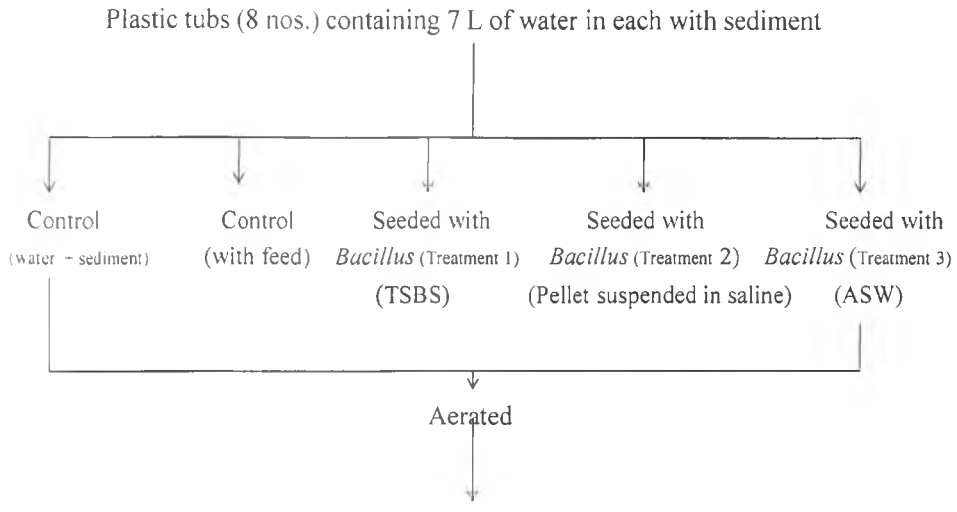


Fig 6: Flow chart of the microcosm experimental set up using *Bacillus* isolate 2 (Expt.2)

Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

To each tub 2 g of finely ground commercial feed was added. The tub water was then allowed to eutrophicate for a week till the level of total NH_3 increased substantially (Plate 2). Out of the 8 tubs, 2 served as control: one without addition of any feed and the second with addition of feed. The selected bacterial isolates to be tested for their efficacy to reduce NH_3 were grown individually in 10 ml test tubes containing 5 ml TSB. After 2-3 days of incubation, to the treatment tub 1, bacterial cells grown in TSB was added, to treatment tub 2, bacterial cell pellets harvested by centrifuging at 5000 rpm at 3 min in a centrifuge and pellet suspended in saline was added and in treatment tub 3, bacterial cells grown in ASW (artificial sea water) was added (Fig 5). Routine monitoring of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were carried out daily till NH_3 level reduced significantly.

The experiment was repeated as described above (Fig 6).

3.6. Analysis of water quality parameters

The water and sediment samples were collected aseptically and transported to laboratory in an ice box. The samples were analysed for the water quality parameters such as pH, dissolved oxygen, ammonia, nitrite, nitrate, phosphate and salinity. Dissolved oxygen was analyzed following azide modification of Winklers method (APHA, 1992). pH of water sample was recorded using the digital pH meter (NAINA, India). The salinity of the sample was analyzed by Mohr's method (Strickland and Parsons, 1972) and was recorded as parts per thousand (ppt). Ammonia nitrogen was estimated by phenol-hypochlorite method (Strickland and Parsons, 1972). Bendschneider and Robinson's method was used for reactive nitrite and for estimating nitrate–nitrogen (Strickland and Parsons, 1972) was used. Phosphate - phosphorus was determined by the ascorbic acid method (APHA, 1992)

Results

IV. EXPERIMENTAL RESULTS

4.1. Bacteriological analysis

4.1.1. Isolation of *Bacillus* sp.

80 samples each of water and sediment were analyzed for the presence of *Bacillus* spp. Presumptive colonies of *Bacillus* spp were subjected to a battery of biochemical tests. Out of 80 samples, 40 *Bacillus* isolates were obtained and subjected to anti-vibrio activity (Table 8). Among the 40 isolates only 2 showed good anti-vibrio activities. Two isolates which showed good anti-vibrio activity were used for microcosm experiment.

Spread plate technique was followed for *Bacillus* sp enumeration. The bacterial cells grown in TSBS were suitably diluted using 0.85% physiological saline. 0.1ml of each dilution was spread on TSAS plates and incubated at room temperature (29°±1°C). After incubation bacterial colonies were counted. Total bacterial count of 1.7×10^9 CFU/ml was observed and used for further experiments.

4.2. Analysis of water quality parameters in control tubs in microcosm

In experimental set up 1, the water pH recorded in control tubs on the 3rd day was 7.3 (Table 6 and Fig 7a) and 7.4 on the 6th day, which thereafter peaked to a level of 7.6 on the 10th day. However, in experimental set up 2, the pH which was recorded in control tubs as 8.3 and 8.4 on 3rd and 6th day respectively decreased to 7.7 on the 10th day (Table 7 and Fig 7b). The salinity of water in both control tubs was 35 ppt on 3rd day, in experiment 1 and 2, which increased to 45 ppt in experiment 1 and 40 ppt in experiment 2 on the 6th and 10th day respectively (Tables 6, 7 and Figs 8a, 8b). The increase in salinity was mainly due to

evaporation. Dissolved oxygen level on 3rd day ranged from 5.2-5.3 mg/l. However, the level of dissolved oxygen was observed to decrease steadily from the 3rd day onwards and was recorded to be 0.81 mg/l, 2.40 mg/l on 6th day and optimum level of 2.0 mg/l on 10th day in experiment 1 (Table 6 and Fig 9a). Similar observation was made for experiment 2, where in the initial level of 5.0-5.1 mg/l decreased to 1.0-2.2 mg/l on the 6th day and 1.5-2.5 mg/l in experiment 2 on 10th day (Table 7 and Fig 9b). The nutrient level of ammonia which ranged from 0.386-0.434 mg/l on 3rd day (Table 6 and Fig 10a) and 0.095-0.827 mg/l on the 6th day was observed to reach a maximum level 1.620 mg/l, on the 10th day in experiment 1. In experiment 2, the minimum level ranged from 0.240-0.228 mg/l on 3rd day (Table 7 and Fig 10b), and level of 0.11-0.664 mg/l on the 6th day and maximum level of 0.732 mg/l on the 10th day. In experiment 1, nitrite level ranged from 0.12-0.20 mg/l on 3rd day in control tubs. A steady decreased in nitrite level was observed on 10th day, it was 0.1 mg/l in both control tubs (Table 6 and Fig 11a). In experiment 2, the nitrite level was 0.14 mg/l on 3rd day, however, on the 6th day the levels was seen to increase and ranged from 0.18-0.22 mg/l. On the 10th day the values recorded showed a decrease in nitrite level it was 0.16-0.20 mg/l (Table 7 and Fig 11b). The minimum nitrate level ranged from 1.0-1.2 mg/l on 3rd day in control tubs, however, rose to a maximum level (1.2 mg/l) on the 6th day and optimum level (1.2 mg/l) on 10th day in experiment 1 (Table 6 and Fig 12a). In experiment 2, minimum nitrate level ranged from 1.0-1.2 mg/l on 3rd, 6th and 10th day in control tubs (Table 7 and Fig 12b). The minimum phosphate level was 0.05 mg/l on the 3rd day in control tubs in experiment 1 (Table 6 and Fig 13a), however, on the 6th day it ranged from 0.05-0.50 mg/l, and reached an optimum level of 0.53 mg/l on the 10th day. Similarly in microcosm experiment 2, the phosphate levels recorded on 3, 6, 10th day was 0.05-0.06 mg/l, 0.05-0.21 mg/l and 0.07-0.1 9 mg/l respectively.

Table 6: Physico- chemical parameters and nutrient concentration of water during microcosm experiment (Expt.1)

Days	Parameters	Control (water+sediment)	Control +feed	Treatment tubs treated with *Bacillus* isolate 1		
				Treatment 1	Treatment 2	Treatment 3
3	pH	7.3	7.2	7.3	7.2	7.2
	Salinity (mg/l)	35	35	35.5	36	36
	Dissolve oxygen (mg/l)	5.2	5.3	5.7	5.4	5.6
	Ammonia (mg/l)	0.386	0.434	0.33	0.29	0.15
	Nitrite (mg/l)	0.20	0.12	0.1	0.1	0.1
	Nitrate (mg/l)	1.2	1.0	1.2	1.0	1.1
	Phosphate (mg/l)	0.05	0.05	0.14	0.12	0.1
6	pH	7.3	7.4	7.4	7.4	7.4
	Salinity (mg/l)	39	39	41	43	45
	Dissolve oxygen (mg/l)	2.40	0.81	1.41	2.0	1.62
	Ammonia (mg/l)	0.095	0.827	1.59	1.68	1.28
	Nitrite (mg/l)	0.20	0.14	0.14	0.13	0.16
	Nitrate (mg/l)	1.2	1.2	1.4	1.3	1.4
	Phosphate (mg/l)	0.05	0.50	0.52	0.34	0.49
10	pH	7.6	7.6	7.6	7.6	7.6
	Salinity (mg/l)	40	40	45	45	45
	Dissolve oxygen (mg/l)	2	2	2.5	2.5	2.5
	Ammonia (mg/l)	0.086	1.620	1.20	1.24	1.01
	Nitrite (mg/l)	0.18	0.14	0.1	0.1	0.1
	Nitrate (mg/l)	1.2	1.2	1.2	1.0	1.0
	Phosphate (mg/l)	0.05	0.53	0.25	0.14	0.15

Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Table 7: Physico- chemical parameters and nutrient concentration of water during microcosm experiment (Expt.2)

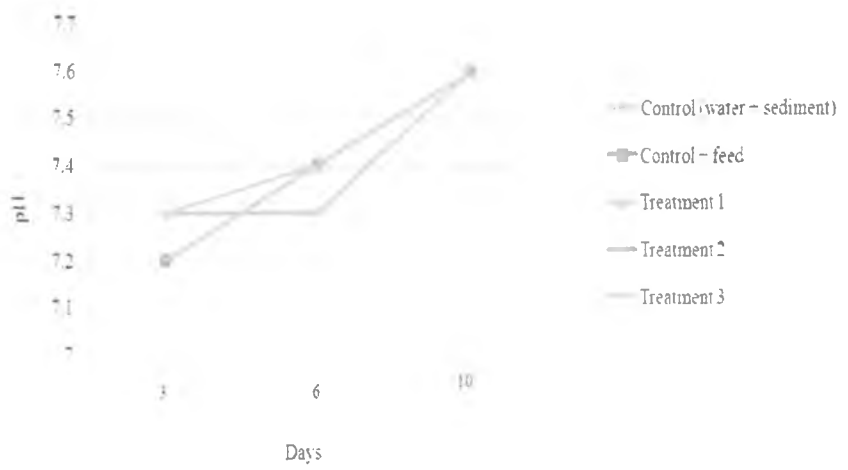
Days	Parameters	Control (water+sediment)	Control +feed	Treatment tubs treated with *Bacillus* isolate 2		
				Treatment 1	Treatment 2	Treatment 3
3	pH	8.3	8.3	8.0	8.2	8.1
	Salinity (mg/l)	35	35	35.5	36	36
	Dissolve oxygen (mg/l)	5.0	5.1	5.4	5.4	5.4
	Ammonia (mg/l)	0.228	0.240	0.27	0.28	0.19
	Nitrite (mg/l)	0.14	0.14	0.16	0.13	0.12
	Nitrate (mg/l)	1.2	1.0	1.2	1.0	1.1
	Phosphate (mg/l)	0.05	0.06	0.11	0.1	0.06
6	pH	8.4	8.4	8.3	8.4	8.3
	Salinity (mg/l)	38	38	39.5	40	40
	Dissolve oxygen (mg/l)	2.2	1.0	1.3	2.0	1.4
	Ammonia (mg/l)	0.11	0.664	0.96	1.35	1.41
	Nitrite (mg/l)	0.18	0.22	0.27	0.23	0.24
	Nitrate (mg/l)	1.2	1.2	1.5	1.5	1.6
	Phosphate (mg/l)	0.05	0.21	0.23	0.29	0.38
10	pH	8.0	7.7	7.7	7.7	8.0
	Salinity (mg/l)	40	40	45	45	45
	Dissolve oxygen (mg/l)	2.5	1.5	2.6	2.8	3
	Ammonia (mg/l)	0.10	0.732	0.50	0.47	0.16
	Nitrite (mg/l)	0.16	0.20	0.18	0.13	0.13
	Nitrate (mg/l)	1.2	1.3	1.3	1.1	1.0
	Phosphate (mg/l)	0.07	0.19	0.1	0.16	0.13

Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 7a: pH levels at different stages of experiment 1 in microcosm study

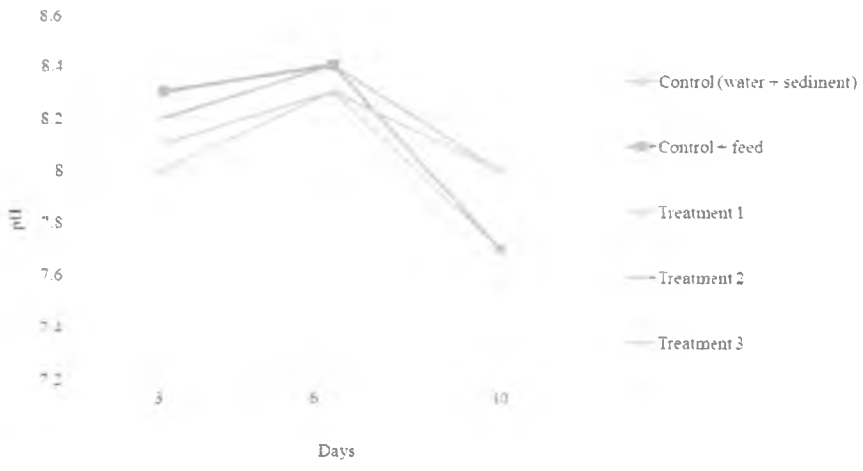


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 7b: pH levels at different stages of experiment 2 in microcosm study

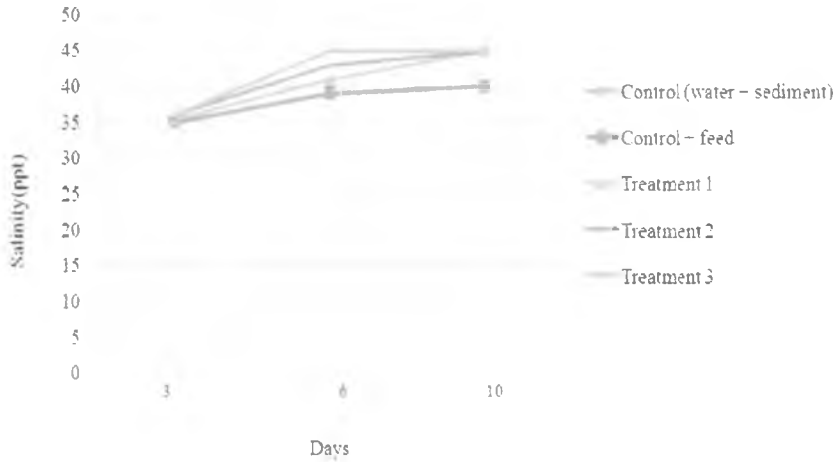


Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 8a: Salinity at different stages of experiment 1 in microcosm study

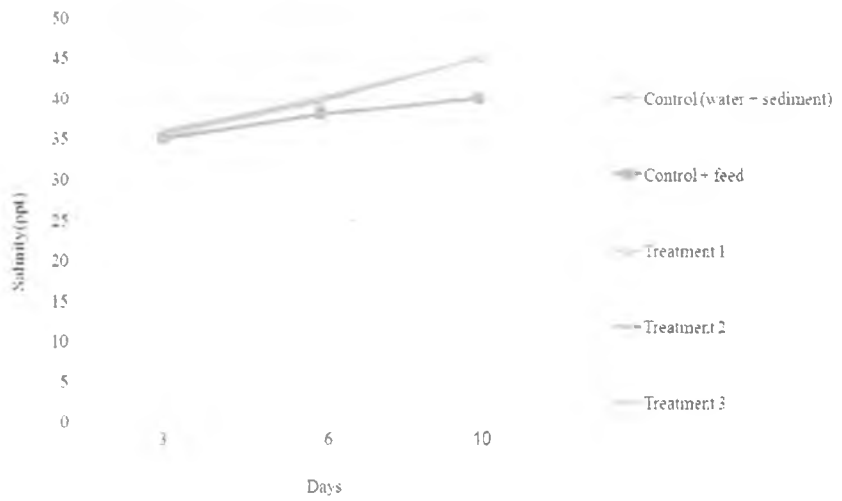


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 8b: Salinity levels at different stages of experiment 2 microcosm study

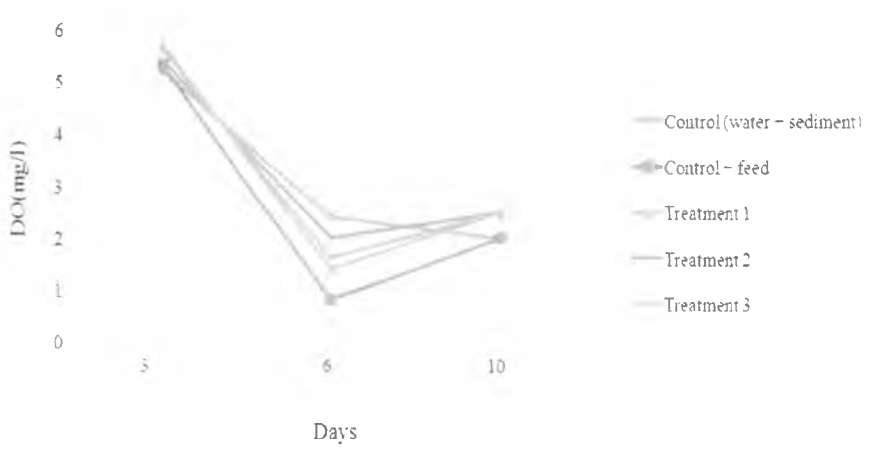


Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 9a: Dissolved oxygen levels at different stages of experiment 1 in microcosm study

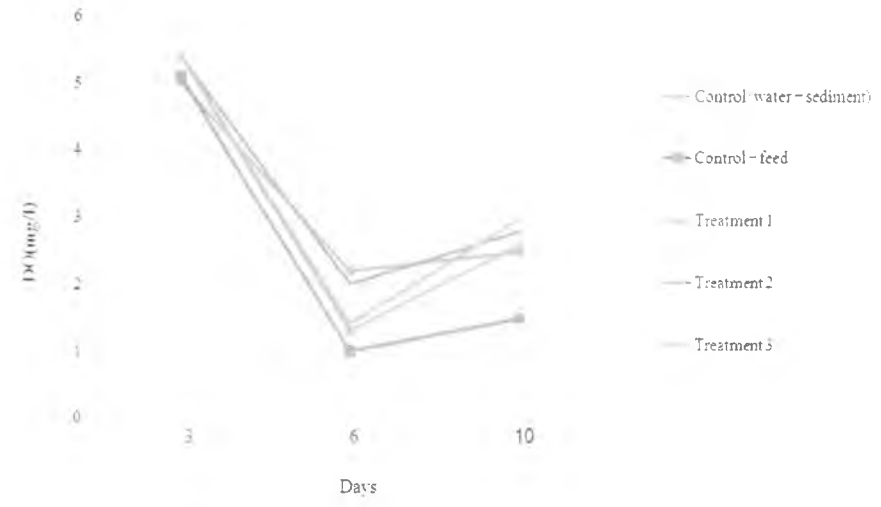


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 9b: Dissolved oxygen levels at different stages of experiment 2 in microcosm study

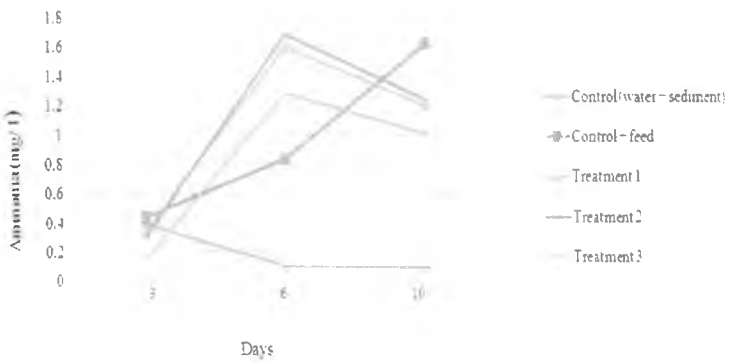


Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 10a: Ammonia concentration at different stages of experiment 1 in microcosm study

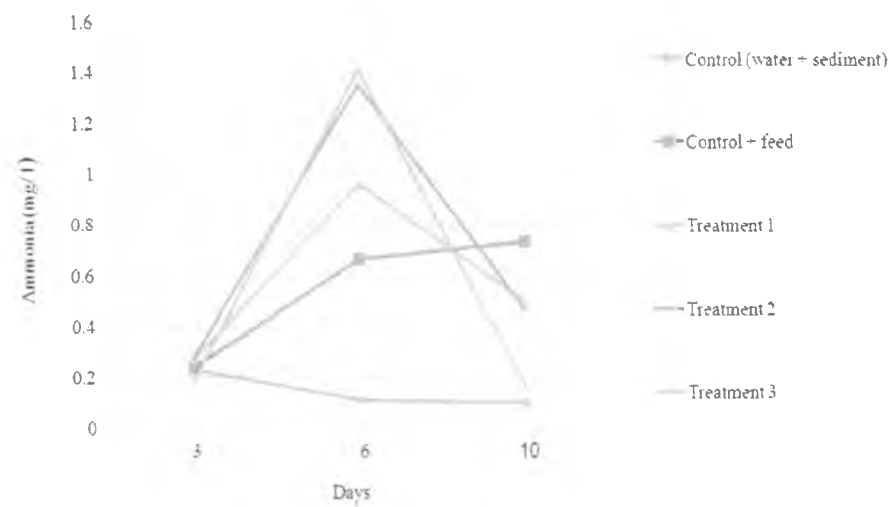


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 10b: Ammonia concentration at different stages of experiment 2 in microcosm study

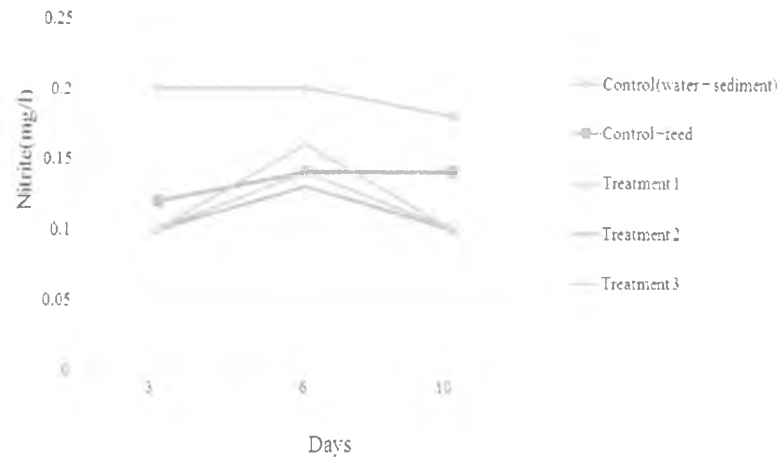


Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 11a: Nitrite concentration at different stages of experiment 1 in microcosm study

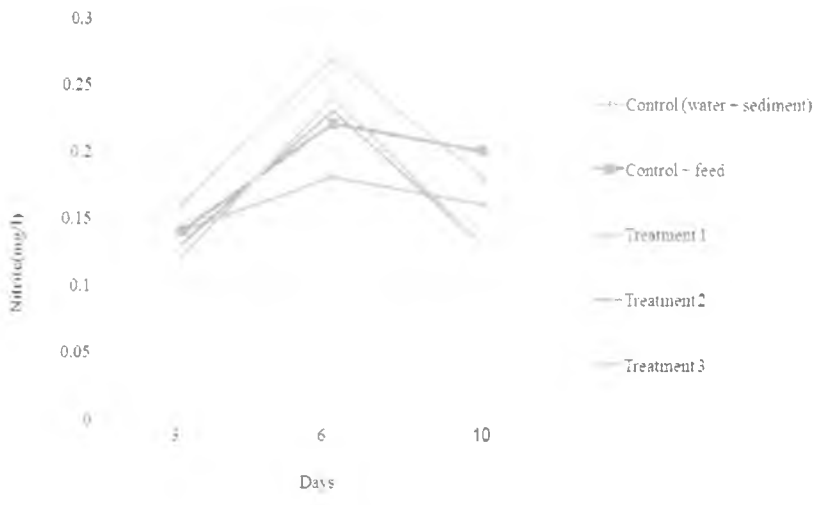


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 11b: Nitrite concentration at different stages of experiment 2 in microcosm study

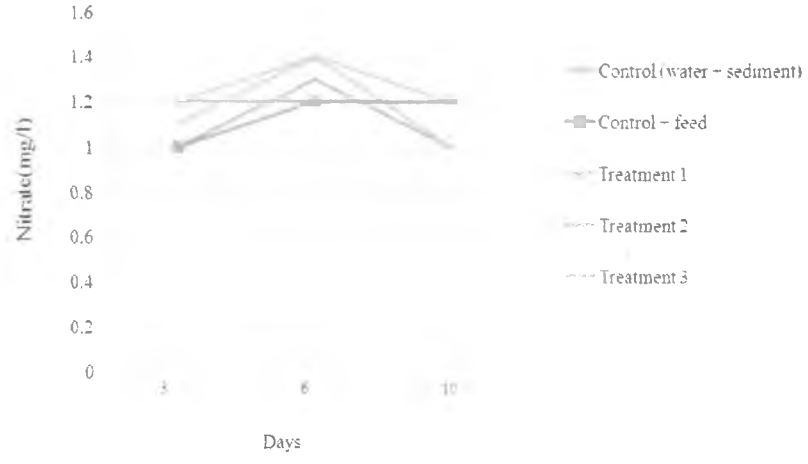


Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 12a: Nitrate concentration at different stages of experiment 1 in microcosm study

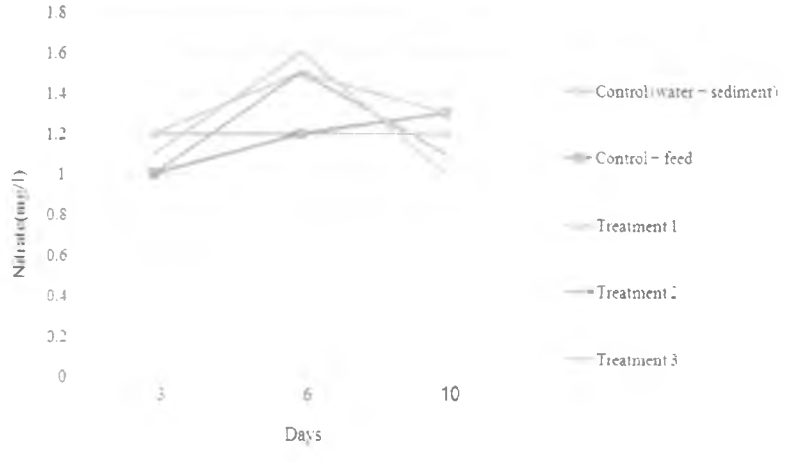


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 12b: Nitrate concentration at different stages of experiment 2 in microcosm study

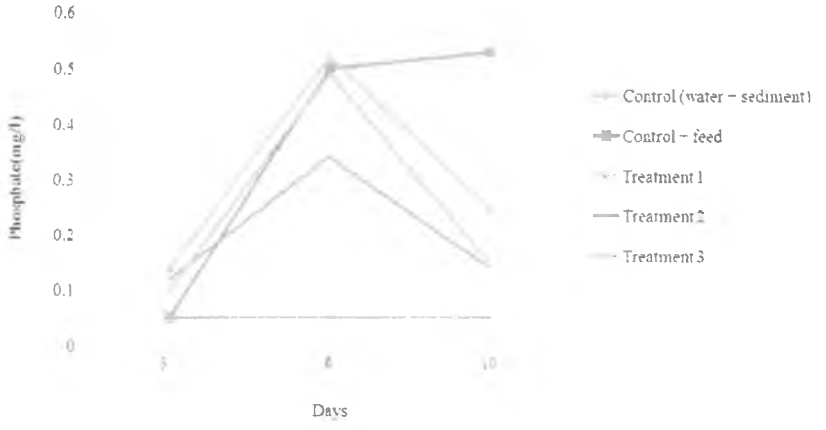


Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

Fig 13a: Phosphate concentration at different stages of experiment 1 in microcosm study

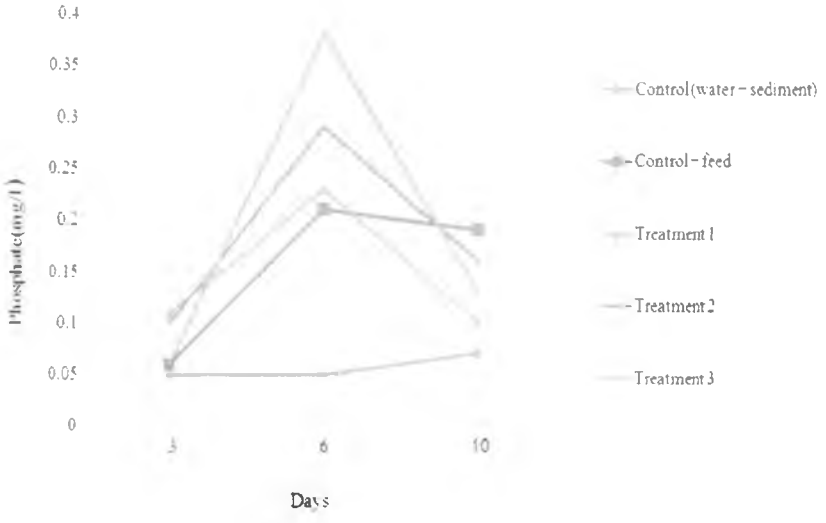


Treatment 1: *Bacillus* isolate 1 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 1 suspended in saline

Treatment 3: *Bacillus* isolate 1 grown in ASW (artificial sea water)

Fig 13b: Phosphate concentration at different stages of experiment 2 in microcosm study



Treatment 1: *Bacillus* isolate 2 grown in TSBS (trypticase soya broth)

Treatment 2: Pellet of *Bacillus* isolate 2 suspended in saline

Treatment 3: *Bacillus* isolate 2 grown in ASW (artificial sea water)

4.3. Water quality parameters in microcosm experiments after seeding with *Bacillus* sp.

Ammonia, nitrite, nitrate, and phosphate levels reduced in experimental tubs inoculated with *Bacillus* sp. The selected bacterial isolates were grown in TSAS before start of the experiment. In the treatment 1, *Bacillus* grown in TSB was added; in treatment 2, bacterial cell pellets, while in treatment 3 bacteria grown in ASW (artificial sea water) was inoculated in both experiment (Experiment 1 and 2). The volume of each application of culture was adjusted and added individually to each tub such that the final density attained was 1.7×10^9 CFU/ml. After inoculation of *Bacillus* culture into different treatments, water quality parameters were analyzed at set intervals. In the present study, the pH of water in microcosm experimental set up was in the range of 7.2-7.6 in all the treatments of experiment 1 (Table 6), and minimum pH (7.2) recorded on 3rd day in experiment 1, where as in experiment 2 pH ranged from 7.7-8.0 in all treatments (Table 7). A steady decrease in pH level observed on 10th day was 7.7 in experiment 2 (Table 7). The highest level of dissolved oxygen 5.7 and 5.4 mg/l was recorded on 3rd day of experiment 1 and experiment 2 respectively (Table 6, 7). Decrease in dissolved oxygen to a minimum level of 1.41 mg/l (Table 6) in experiment 1 and 1.3 mg/l (Table 7) in experiment 2 on 6th day was recorded. The maximum salinity of 45 ppt and a minimum of 35 ppt were recorded on 10th day and 3rd day respectively in both the experiment (Table 6 and 7). The level of ammonia was ranged from 0.095-1.68 mg/l and 0.11-1.41 mg/l in experiment 1and experiment 2 respectively on 6th day (Table 6, 7). The highest nitrite levels was recorded on 6th day, was 0.13-0.20 mg/l (Table 6) however, on 10th day nitrite level decreased and ranged from 0.1-0.18 mg/l in experiment 1 and level of 0.13-0.20 mg/l in experiment 2 (Table 7), the low nitrite level of 0.1-0.20 mg/l recorded on 3rd day in experiment 1 and 0.12-0.16 mg/l in experiment 2 and at the end of the experiment there is a

steady decreased in nitrite level in both experiments. The low nitrate level of 1.0-1.2 mg/l on 3rd day in all treatments in both experiments (Table 6, 7) and maximum was 1.2-1.4 mg/l in experiment 1 (Table 6) and 1.2-1.6 mg/l in experiment 2 (Table 7) on 6th day. However, on 10th day nitrate level decreased, ranging from 1.0-1.2 mg/l in experiment 1 and level of 1.0-1.3 mg/l in experiment 2. Phosphate concentration in experiment 1, ranged from 0.05-0.1 mg/l (Table 6), where as in experiment 2, it was in the range of 0.05-0.11 mg/l in all treatments on 3rd day (Table 7). An increased level of phosphate was observed on addition of feed on 6th day and thereafter it reduced to a lowest level on 10th day. In the present study, the bacteria grown in ASW showed better result in clearing the blackening of soil bottom when compared to the bacterial culture grown in TSB and cell pellets in both experiments (Experiment 1 and 2) (Plate 3).

Plate 4: Plate showing clearing zone by *Bacillus* sp in starch agar

Table 8: Prevalence of *Bacillus* sp. in Netravati estuary

Sampling No. (Netravati estuary)	No. of sample positive for *Bacillus* in samples analysed	
	Sediment	Water
	No. positive/Total analysed	No. positive/Total analysed
1	4/5	1/5
2	3/5	2/5
3	3/5	2/5
4	2/5	1/5
5	4/5	3/5
6	3/5	2/5
7	3/5	2/5
8	3/5	2/5
Total	**25/40**	**15/40**

Discussion

V. DISCUSSION

5.1. Bacteriological analysis

In the present study, 40 water and 40 sediment samples, were analyzed. A total of 40 *Bacillus* spp were isolated from 80 samples, with majority of them from sediment (Table 8). This could be attributed to the fact that sediment receives high levels of nutrient and thus provides more nutrients and better shelter to both planktonic as well as biofilm forming bacteria. The higher total plate counts of bacteria recorded in sediment than in water sample could be due to the above reasons.

5.2. Sample analysis

5.2.1. Analysis of water quality parameters in microcosm experiment

Water quality parameters such as pH and salinity were high throughout the experiments. In experiment 2, pH decreased on 10^{th} day and was 7.6 (Table 7).

In experiment 1 pH was in the range of 7.2-7.6, whereas in experiment 2, it was 7.7-8.0. The pH values of both experiments were near the optimum value of 7.5- 8.7 which is required for shrimp culture as reported by MPEDA, (1992) and below the values reported by Briggs and Funge- Smith (1994) in intensive shrimp farms of Thailand. The pH values in this study were in the range (7.09-8.5) which in agreement with the report of Sheryl *et al.* (2010). The pH values observed in this study was nearer to the optimum pH (6.8-8.7) which is required for the maximum growth and production of shrimp (Ramanathan *et al.,* 2005; Ramakrishan, 2000; Soundarapandian and Gunalan, 2008). The lowest value of pH 7.2 was recorded on 3^{rd} day in experiment 1 and 8.0 in experiment 2. At the end of experiment (10^{th} day), pH was 7.6 in both

the experiments (Table 6, 7). The rise in pH after addition of feed could be due to the generation of ammonia and amines from the nitrogenous components of the feed. However, the gradual decline seen after seeding with *Bacillus* species could be due to production of acids by fermentation and due to the ability of this group of bacteria to oxidize ammonia to nitrite and nitrate.

The salinity in the present study ranged from 35 to 45 ppt and highest level of 45ppt were observed on the 10^{th} day in both the experiments (Table 6, 7). Marginal increases in salinity were seen over the period in both experiments. This increasing trend of salinity could be due to evaporation. Muthu (1980), Soundarapandian and Gunalan, (2008), Karthikeyan (1994) recommended a salinity range of 10-35 ppt to be ideal for *P. monodon* culture. A salinity value in the range of 7.20-43.18 was reported by Sheryl *et al.* (2010).

Dissolved oxygen levels were reported to be in the range of 4.06 - 4.81 mg/l in aerated and non-aerated ponds by Sheryl *et al.* (2010). A decrease in dissolved oxygen reaching a minimum of 2.93-2.86 mg/l was observed in both aerated and non-aerated ponds by same workers. After addition of feed, minimum value of dissolved oxygen recorded on 6^{th} day, was 0.81-2.40 mg/l in experiment 1 (Table 6) and 1.0-2.2 mg/l in experiment 2 (Table 7). After addition of feed it declined drastically, probably due to increased activities of proteolytic bacteria resulting in the break down of feed, and formation of reducing substances which utilize the dissolved oxygen. The growth of sulphate reducers was probably facilitated resulting in sediment blackening (Moriarty, 1997).

Ammonia levels on 3^{rd} day ranged from 0.150 mg/l to 0.434 mg/l and 0.190 to 0.280 mg/l in experiment 1 and 2 respectively. After addition of feed, from the 6^{th} day onwards, the level increased to 1.68 mg/l in experiment 1 and 1.41 mg/l in experiment 2. In the present study

high level of ammonia were observed before addition of *Bacillus*. This may be due to break down of feed particles leading to the production of reducing substances. After treatment with *Bacillus* the level of ammonia was reduced drastically. The result of this study is in agreement with the observation of several workers (Moriarty, 1997; Rao and Karunasagar, 2000). The permissible level of ammonia-N has been reported to be < 1 mg/l in shrimp culture (MPEDA, 1992). In our study, ammonia level was well below the permissible limit i.e. 0.16 mg/l in experiment 2 (Table 7) and 1.01 mg/l in experiment 1 after addition of *Bacillus* (Table 6). It is surmised that heterotrophic bacteria like *Bacillus* utilize ammonia as the main source of nitrogen for their growth. There was a marginal reduction in total ammonia level even in the control tubs in the study which could be due to activity of natural bacteria and/or oxidation of ammonia by continuous agitation by aerators in the small water column (Chiyavaressaja and Boyd, 1993, Rao, 1997).

According to MPEDA, a nitrite level of 0.25 mg/l is recommended in shrimp culture (MPEDA, 1992). In the present study, low levels of nitrite were observed after the addition of *Bacillus* sp. the levels of nitrite observed (0.2 mg/l in experiment 1 and 0.16 mg/l in experiment 2), in this study in agreement with the report of Sheryl *et al.* (2010) where low levels (0.28 mg/l) nitrite was found throughout the cycle in the aerated ponds. The low level of nitrite in our study could be due to the oxidation of nitrite to nitrate by the added heterophilic *Bacillus* and continuous aeration.

The nitrate concentration in the pond is also generally high throughout the cycle in the aerated ponds (4.72 mg/l) as report of Sheryl *et al.* (2010). The level of nitrate in our study was found to be constant throughout the experiment (1.2-1.46 mg/l) in experiment 1 and 1.2-1.6 mg/l in experiment 2. Estuaries have wastes from both natural and anthropogenic origins.

Nitrates found in leaf litter, organic debris, and nutrient-rich soils by natural processes enter our riverine systems from runoff during rain events. Some nutrients enter the estuary from the ocean; however, their total contribution is much less than that of the fresh water runoff. All these sources contribute to the natural levels of nitrate in the sea water.

In the present study, phosphate level was in the range of 0.05-0.52 mg/l in experiment 1, and 0.05-0.38 mg/l in experiment 2. Phosphate values for both experiments were high compared to the values reported by Briggs and Funge-Smith (1994). It is possible that the high phosphate levels is a result of discharge of sewage and other wastes which if unutilized or underutilized, accumulate in a farm situation. Low level phosphate solubilizers in the tubs could have been responsible for the high levels of phosphate.

5.3. Ammonia reduction in microcosm

5.3.1. Reducing ammonia levels in relation to other water quality parameter

Excess application of nitrogenous fertilizers to the pond leads to accumulation of ammonia and nitrite. The toxic form of nitrogen compound ammonia and nitrite can cause mass mortality of aquaculture animals in a closed culture system (Chin and Chen, 1986). Therefore there is a need to reduce the amount of ammonium in the pond so as to increase survival rate as well as productivity of cultured animals. Several management practices have been suggested to reduce ammonium concentration in ponds such as regular water exchange, regulating feed and feeding practices, water circulation and aeration, decreasing pond depth and bioremediation (Hargreaves, 1998).

During this study, we have evaluated the bioremediation potential of *Bacillus* sp to reduce ammonia levels in microcosm environment. In the present study, reduced levels of ammonia

were observed in both experiments treated with *Bacillus* sp (1.01 mg/l in experiment 1 and 0.16 mg/l in experiment 2) with relatively high concentration in unseeded control. The results present evidence that *Bacillus* sp. were more effective in reducing the total ammonia level as in the experimental set up compared to unseeded control. It is quite possible that under certain condition *Bacillus* sp may also utilize ammonia for their growth. Under farm condition, the ammonia level should be less than 1 mg/l. ammonia levels observed in this study were well below recommended level. The result of this study is full agreement with the earlier published reports (Moriarty, 1996; Anon, 1995). Most of the commercial bioremedial products contain *Bacillus* and have been reported to be effective in farm condition. A marginal reduction in total ammonia level also seen in unseeded control could be due to activity of the natural microflora and/or the volatilization of ammonia by continuous agitation by aerators in the small water column (Chiyavaressaja and Boyd, 1993; Rao, 1997). Sohier and Bianchi (1985) reported a bacterial density of 5 to 6 $\times 10^6$ CFU/ml as sufficient to develop a heterotrophic bacterial community within a closed aquaculture system. In the present study, *Bacillus* was inoculated at 1.7×10^9 CFU/ml and was effective in reducing ammonia to the allowable limit.

Bacillus sp used in this study able to reduce ammonia to the maximum limit which is allowable in aquaculture systems. *Bacillus* strains had less complex metabolic pathways than autotrophs and can able to both nitrify and denitrify. This makes *Bacillus* strains attractive candidate species for application in the removal of ammonia nitrogen from aquaculture system and wastewater. The *Bacillus* strains isolated in this study seemed to have some effect on reduction of ammonia levels and thus, it can be used in aquaculture systems to efficiently maintain low levels of ammonia throughout the culture period.

Summary

VI. SUMMARY

In the present study, 80 samples (40 sediment and 40 water) were collected from Netravathi estuary from which 40 *Bacillus* isolates were obtained. The *Bacillus* isolates were tested for their bioremediation potential in microcosm set up.

The results are summarized as follows:

1. The load of *Bacillus* sp. was higher in sediment than in water samples
2. *Bacillus* isolates were found capable of utilizing ammonia as main source of nitrogen for their growth and thus reduced the toxicity by keeping ammonia and other chemical parameter of water low compared to unseeded control in microcosms.
3. *Bacillus* cells grown in ASW (artificial sea water) showed better efficacy in reducing ammonia as compared to the cells grown in TSB (trypticase soya broth) that were harvested by centrifuging and used for the study.
4. 1.7×10^9 CFU/ml of *Bacillus* was found to be optimum for application since it gave highest reduction of ammonia and other chemical parameters of water.

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Abstract

VIII. ABSTRACT

Aquaculture, in India, has made progress in the past two decades leading to augmentation of food production, income and employment generation. Increased production is being achieved by expansion of culture areas and the use of scientific methods. However, the wastes consisting of metabolic byproducts, residual food and faecal matter generated in the aquaculture system not only affects the cultured animal but also the quality of natural fauna and flora when these wastes are discharged. Hence, it becomes necessary to use approaches that would help manage wastes. To maintain healthy ecosystem in aquaculture ponds and hatchery tanks bioremediation is a novel potent biotechnological approach where application of microbes into aquaculture ponds is carried out. This study was carried out to evaluate marine *Bacillus* spp. as potential bioremediator reducing toxic substances in microcosm simulated ecosystem. In the present study, 80 samples (40 sediment and 40 water) were collected from Netravathi estuary and 40 *Bacillus* isolates were obtained. Most of *Bacillus* was isolated from sediment samples. Water quality parameter such as pH and salinity were high in both experiments, the pH of water in experiment 1 varied from 7.2-7.6, where as in experiment 2, it ranged from 7.7-8.0. Water salinity during the experimental period ranged from 35- 45 ppt in both experiments. The ammonia level reached a high on 6^{th} day it was 1.68 mg/l in experiment 1, where as in experiment 2 it was 1.41 mg/l. Ammonia levels declined drastically from 10^{th} day of *Bacillus* inoculation from 1.68 mg/l to 1.01 mg/l in experiment 1, and 1.41mg/l to 0.16 mg/l in experiment 2. The nitrate level was high in both the experiments, 1.2-1.4 mg/l in experiment 1, and in experiment 2 it was 1.2-1.6 mg/l. Where as nitrite and phosphate level were too low in both experiments. In the present study, *Bacillus* inoculated at

a density of 1.7×10^9 CFU/ml had shown relative increase in rate of ammonia reduction in different treatments and bacterial cells grown in ASW (artificial sea water) showed better result compared to the bacterial cells grown in TSB and cell pellets harvested by centrifugation in both experiments. Overall experimental result clearly reveals the fact that *Bacillus* sp. has got better efficacy in reducing the total ammonia level and water quality parameter compared to unseeded control.

MARINE *BACILLUS* SPECIES AS BIOREMEDIATORS IN AQUACULTURE PONDS

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