DEVELOPMENT AND EVALUATION OF BIOREMEDIAL MEASURES FOR SHRIMP AQUACULTURE

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in

Fishery Pathology and Microbiology

By

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CERTIFICATE

This is to certify that the work recorded in the thesis entitled ***"DEVELOPMENT AND EVALUATION OF BIOREMEDIAL MEASURES FOR SHRIMP AQUACULTURE"*** submitted by **Sri. Shubhadeep Ghosh** in partial fulfilment of requirement for the Degree of **Master of Fishery Science (Fishery Pathology and Microbiology)** in the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, is the faithful and bonafide research work carried out under my supervision and guidance. The results of the investigation reported in this thesis have not so far been submitted for any other Degree or Diploma. The assistance and help received during the course of investigation have been duly acknowledged.

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We, the undersigned, having satisfied with the performance of **Mr. Shubhadeep Ghosh** in the viva-voce examination, conducted today, the 11th September 2002, recommend that the thesis be accepted for award of the degree.

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Chapter - 1

INTRODUCTION

1. INTRODUCTION

Aquaculture plays a vital role in world economy today and it is fast emerging as a major food industry. Aquaculture is the only hope of meeting the growing need for fish for the increasing world population, as the yield from capture fisheries have come to a stagnancy. Of all the kinds of aquaculture practices, the outlook for shrimp aquaculture appears quite promising all over the tropical and subtropical countries. Asia holds a predominant position in the world shrimp production by culture. This has been possible due to the spectacular technology development and the favourable environment for the utilization of farming technology in the South East Asia over the last 10 to 15 years.

In India the total shrimp production in 2000 was 1,59,000 mt (official estimate 97,096 mt) (Anon, 2001). In West Bengal, the development of coastal aquaculture is centered on shrimp farming. The dominant species under culture is *Penaeus monodon* due to its high unit value realization and ever expanding export demand. The scientific culture of shrimp started in West Bengal during late 1980's and more than 34,600 ha of area was brought under culture. The estimated production of shrimp through aquaculture increased from 12,500 t during the year 1990-91 to 23,445 t in 1995-96. However, the rapid growth of shrimp farming industry halted suddenly in 1996-97. The production during 1999-2000 dropped to 19,960 t (Upadhyay, 2001). The set back to the industry was attributed mainly to environmental and health problems resulting in the outbreak of diseases.

Microorganisms play a major role in cleaning up the environment through rapid mineralization of organic matter present in culture ponds. Generally, in pond environment the organic matter content will be high compared to natural environment due to extraneous input like feed, excreta, fertilizer, etc. The microorganisms present in pond such as bacteria, fungi, protozoa etc., carry out active decomposition of left over feed and metabolites to inorganic forms such as

ammonia, hydrogen sulphide, carbon dioxide etc., through the process of mineralization. These nutrients will be utilized by algae for their growth and in turn produce oxygen, which the microorganisms need for decomposition of organic matter. Such a natural process is called 'self purification' process (Anon, 1993). In a way microorganisms and algae exist as symbiotic partners in ponds.

Many a times the appropriate species of microorganisms for purifying water/sediment and appropriate physcio-chemical conditions may not be always present in the pond to promote rapid growth and speedy mineralization. In this situation, seeding of microorganisms or manipulation of microflora could hasten the mineralization process and bringing about rapid purification. The term "Bioremediation" can be used to describe the process of reducing the hazardous organic wastes to environmentally safe levels through use of micro/macro organisms in ponds. Bioremediation can broadly be classified into Engineered and Intrinsic bioremediation. Engineered bioremediation can again be divided into biostimulation and bioaugmentation (Atlas and Unterman, 1999). Few of the microorganisms, which help in this process, are bacteria like *Bacillus* sp, *Acinetobacter* sp, *Pseudomonas* sp, *Nitrosomonas* sp, *Rhodopseudomonas* sp, etc. As the microorganisms are fast growing (shorter generation time) and bring down levels of toxic products such as NH_3, H_2S etc, to insignificant levels, they are preferred over macroorganisms like algae, mussels, sea cucumber etc.

A variety of commercial bioremediators have been used in shrimp aquaculture to increase shrimp productivity but with varying degrees of success. There are a number of reports on the positive and negative effects of the use of bioremediators in shrimp culture ponds (Boyd *et al.,* 1984; Moriarty, 1996).

The significance of special groups of microorganisms with varied physiological characteristics in shrimp grow-out systems of India is not well documented and so also their ecology in grow-out ponds. Such reports are virtually absent and never been attempted earlier in West Bengal.

The present study was, therefore, taken up with the following objectives

i) To monitor the levels of bacteria of different physiological characteristics involved in nutrient cycling in shrimp culture systems of West Bengal.

ii) To develop and evaluate suitable bioremedial measures using indigenous microflora of shrimp farming system.

iii) To compare the efficacy of bioremediators with indigenous microflora and commercial bioremediators under laboratory conditions.

Chapter - 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Microbial productivity in shrimp culture systems

Microorganisms not only act as autotrophs (primary producers) but also as saprophytes and heterotrophs, thereby helping in rapid recycling of dead and decaying animals and plants (organic matter), which in turn keeping the aquatic ecosystem alive.

The autotrophic community is limited to few photo and chemoautotrophic bacteria, diatoms and cyanobacteria. Photoautotrophic bacteria belonging to the group green sulphur bacteria and purple sulphur bacteria help in carbon dioxide (CO_2) fixation but require anaerobic conditions, sufficient light and hydrogen donors like hydrogen sulphide (H_2S) and organic acid. Chemoautotrophic bacteria like nitrifying bacteria, bacteria involved in sulphur cycle, iron and manganese cycle contribute to primary production to a small extent (Rheinheimer, 1992).

Heterotrophic microorganisms consisting of bacteria and fungi, help in degradation of organic matter, if optimum conditions are prevalent, to simpler forms like CO_2 and inorganic salts.

A quantitative estimation of heterotrophic bacterial counts (THC) in water and sediment of semi-intensive shrimp culture ponds revealed a count up to $3x10^8$ colony forming units (cfu)/g in pond sediments during the entire culture period and a count up to $6x10^6$ cfu/ml in pond water during the culture period (Sharmila, *et al.*, 1996; PremAnand *et al.*, 1996). The higher count in sediments was attributed to continuous availability of substrate and nutrients in the form of unconsumed feed, shrimp excreta, dead plankton and other inorganic and organic matter on pond bottom. The heterotrophic bacterial counts in various shrimp culture systems of West Bengal revealed the count of 4×10^3 cfu/ml – $6x10^6$ cfu/ml in pond water and $7x10^3$ cfu/g - $3x10^7$ cfu/g in pond sediments during the entire culture period (Sengupta, 2002).

Vibrio sp was the dominant flora of all the farms along the east and west coast of India (Otta *et al.*, 1999) and among them *V. alginolyticus* and *V. harveyi* appeared to be the most important species. *Vibrio* counts ranged from $3.2x10^1$ – $6.2x\ 10^2$ cfu/ml of water in farms along the east coast and $5x10^1$ cfu/ml – $2.8\ x10^4$ cfu/ml of water in farms along the west coast; while luminous bacterial counts (LBC) ranged from 1x10 – 7x10 cfu/ml of water in farms along the east coast and up to $1.9x10^2$ cfu/ml of water in farms along the west coast (Otta *et al.*, 1999). The THC and LBC in the coastal waters in the Arabian sea (0-200 m depth) ranged from 8×10^3/lit – 200×10^3/lit and 1×10^3/lit – 10×10^3/lit, respectively. (Ramaiah and Chandramohan, 1987).

Moriarty (1986) observed that the heterotrophic microbial numbers in the water column were higher in most ponds receiving organic matter (feed pellets and chicken manure) than in control ponds. Most heterotrophic bacteria were between 0.4 and 0.8 µm in diameter and 0.5 and 1.5 µm in length. The average cell volume was 0.14 μm^3 and thus biomass was 30 fg (a total of 546 bacteria measured). Novitsky (1983) showed that the heterotrophic activity in the soil-water interface region was several times greater than that in the water column above and twice as high in the sediment immediately below.

The high heterotrophic microbial productivity coupled with stress and unfavourable environmental conditions, often lead to outbreak of shrimp diseases. Snieszko (1974) described the interaction of these factors in a disease production equation as follows:

$H\ (A+S^2) = D$; where, H = species/strain of host

A = etiological agent

S = environmental stressors

D = disease

Environmental stressors are squared because as shrimp approach adaptation limits, effect of stressors increase geometrically. Also when more than one stressor

is involved [oxygen (O_2), ammonia (NH_3), CO_2, temperature, etc.] detrimental effects are magnified.

2.2. Degradation of organic substances by microbes in shrimp culture systems

Microorganisms efficiently utilize the organic matter to synthesize their cell structure and the energy for their life processes. The breakdown of organic matter or mineralization is the major role played by microorganisms. If microorganisms would not have helped in degradation, the problem due to organic matter pollution would have been magnified. Even though the microorganisms can utilize organic matter, they need optimum conditions such as temperature, pH, O_2, oxidation reduction potential (Eh), proper carbon (C) : Nitrogen (N_2) ratio, etc as these are the major limiting factors for their growth. Organic matter usually contains three main energy nutrients, viz., proteins, carbohydrates and fats.

2.2.1. Proteins

There are plenty of proteolytic microorganisms, which can utilize protein as a source of energy. The decomposition of proteinaceous material to soluble amino acids and other compounds is necessary for assimilation of this material into bacterial protoplasm. The breakdown of protein is also important for the release of nutrients from refractory compounds. Thus, generation of nitrogenous compounds is achieved. *Enterobacter, Pseudomonas* and other eubacteria and various fungi can carry out proteolysis. Proteins are primarily hydrolyzed to polypeptides and oligopeptides by exo-enzymes of microorganisms. Later, they are taken up by cells, broken down, then utilized for bodybuilding, and lastly deaminated with liberation of NH_3. Zobell and Upham (1944) found that out of 60 strains of bacteria tested all could breakdown peptone to NH_3 and 47 could liquefy gelatin.

Sepers (1981) reported on a number of bacteria, which can utilize amino acids as the sole C, N_2 and energy source. According to him, 83% of the tested

organisms were capable of utilizing 50-83% of the applied organic compounds as sole carbon and energy source. The amino acids most resistant to bacteria are methionine, taurine, threonine and glycine.

Barat and Jana (1987) studied the protein mineralizing bacteria and ammonifying bacteria in culture tanks and reported that seasonal changes of protein mineralizing bacteria were less pronounced with relatively low numbers in July than in the remaining months of the year. The ammonifying bacteria showed a small peak in autumn. The increase in intensification of the culture system lead to increased metabolite production which supported higher population of protein mineralizing bacteria (Barat and Jana, 1987).

2.2.2. Carbohydrates

Many eubacteria as well as actinomycetes and numerous fungi are able to degrade simple sugars to 3C compounds and finally to CO_2 and water (H_2O) under aerobic conditions. Under anaerobic conditions only fermentation is possible.

Few bacteria are capable of breaking down disaccharide such as sucrose, lactose and maltose, and polysaccharides such as mannitol, rhammose and xylose. These include *Azotobacter, Desulfovibrio, Clostridium, Klebsiella* and *Enterobacter* (Herbert, 1975; Lakshmanaperumalsamy *et al.*, 1975).

Starch is an important food reserve in plants. It is a polysaccharide, which is utilized by only 10% of bacteria as C, N and energy source (Sepers, 1981). *Pseudomonas, Bacillus,* actinomycetes and higher fungi can hydrolyze starch by means of exo-enzymes (amylase, maltase) into glucose under aerobic conditions, whereas *Clostridium* utilize starch under anaerobic conditions.

Cellulose are decomposed by myxobacteria (*Cytophaga* and *Sporocytophaga*) and higher fungi (ascomycetes and deuteromycetes) under aerobic conditions (Rheinheimer, 1992).

Agar and alginic acids are the product of red and brown algae, respectively and are degraded by the action of many bacteria, viz., *Achromobacter,*

Agarobacterium, Flavobacteruin, Cytophaga, Alginomonas alginovorus, A. alginica and others (Rheinheimer, 1992). Chitin, a skeletal component of many lower animals, fungi and crustaceans are broken down by bacteria of the genera *Pseudomonas, Vibrio* and by fungi (Rheinheimer, 1992).

2.2.3. Fats

Fats are esters of fatty acids with glycerol, contained in plants and animals and also in water and sediment. Zobell and Upham (1944) isolated 13 species of lipolytic bacteria belonging to genera *Pseudomonas, Vibrio, Sarcina, Serratia* and *Bacillus.* Bianchi *et al.* (1992) reported that out of 20 isolates of NH_3 and nitrite (NO_2^-) oxidizing bacteria, 49% and 21% could utilize fatty acids as carbon and energy source.

2.3. Role of microorganisms in nutrient cycles of shrimp culture systems.

Microorganisms help not only in the production and breakdown of organic matter but also in nutrient recycling. Nutrient cycling is an essential process in the aquatic ecosystem. N_2 - cycle, C - cycle, sulphur (S) - cycle and phosphorus (P)-cycle are the major nutrient cycling process going on in the aquatic ecosystem and these play a key role in the formation of organic materials. Carbon is the prime substance of all organic materials; nitrogen is necessary for the synthesis of amino acids, nucleic acids and amino sugars; sulphur is essential in the sulfhydryl groups of amino acids and their polymers and phosphorus is contained in nucleic acid, phosphate esters, sugar phosphates and adenosine triphosphate (Austin, 1988).

2.3.1. Nitrogen cycle

Nitrogen is a major constituent of proteins, the building block of all living matter. The N_2 cycle, therefore, occupies an important place in organic matter

recycling. It involves N_2 fixation, ammonification, nitrification and denitrification processes carried out by different microorganisms.

Biological N_2 fixation transforms molecular N_2 to NH_3 or organic N_2 and through this process, the atmospheric N_2 enters the biosphere and gets involved in N_2 cycle in aquatic environments. It is carried out by prokaryotes referred to as "diazotrophs". Stal *et al.* (1984) citied evidence of N_2 fixation for 18 blue green algae belonging to the genera *Anabaena, Calothrix, Microchaete, Nostoc, Nodularia, Rivularia* and *Trichodesmium.* The occurrence of *Azotobacter, Clostridium, Desulfovibrio* and photosynthetic N_2 fixing bacteria in marine sediments has been documented of which *Desulfovibrio* plays an important role (Sisler and Zobell, 1951; Pschenin, 1963; Truper and Genovese, 1968; Wyne Williams and Rhodes, 1974a, b). The nitrogenase activity is light stimulated and to some degree inhibited by O_2 (Stal *et al.,* 1984).

Green plants utilize NH_3 and nitrate (NO_3^-) as source of N_2 for synthesis of protein (Rheinheimer, 1992). The complex proteinaceous matter is converted to free NH_3 or ammonium ion (NH_4^+) depending on pH first by protein mineralizing bacteria and then by ammonifying bacteria such as *Pseudomonas, Bacillus* and *Vibrio.* This process is called ammonification and is the dominant mechanism for NH_3 production (Fry, 1987). Ammonification can take place either aerobically or anaerobically in water and sediment (Fry, 1987).

Ammonia is also produced from NO_3^- by nitrate dissimilation, which is important in anaerobic sediments. Herbert (1982) showed that *Aeromonas, Vibrio, Klebsiella, Escherichia* and *Clostridium* were very active in NO_3^- dissimilation and they contained an enzyme NO_3^- reductase whose activity reaches maximum under anaerobic conditions.

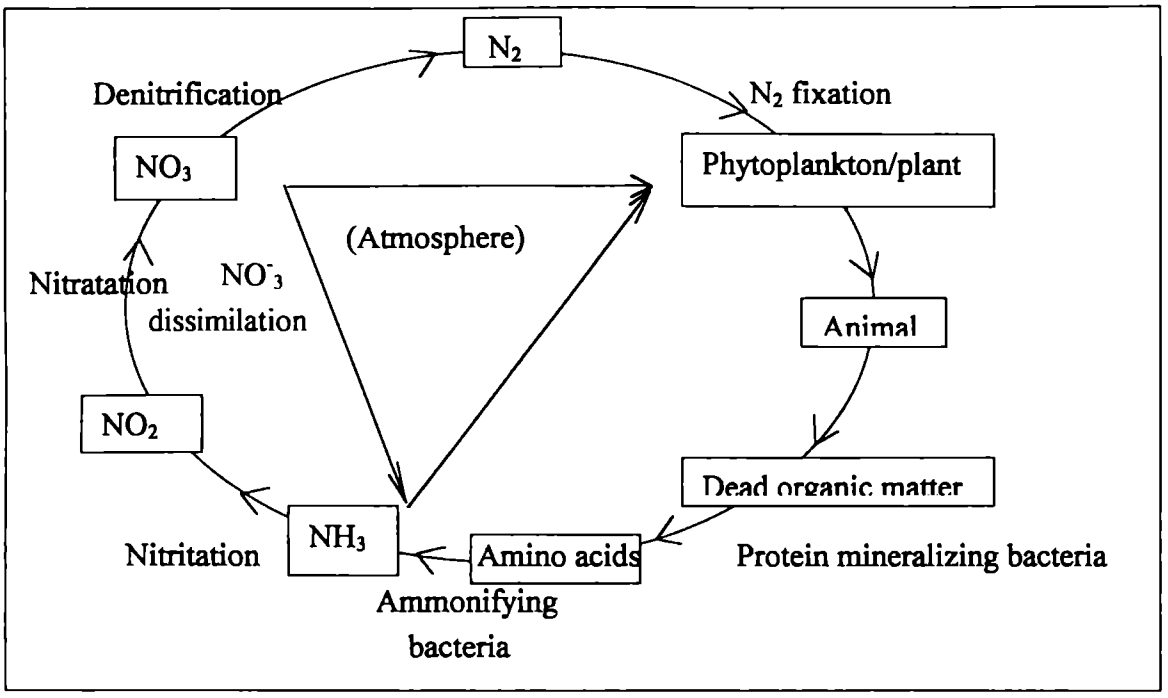
In aerobic sediments and in the water column, NH_3 gets oxidized to NO_3 by nitrification process. The organisms involved in nitrification have been fully described (Watson *et al.,* 1981) and consist of two genera that use different

respiratory mechanisms. The NH_3 oxidizers convert NH_3 to NO_2^- (nitritation); there are 5 genera of which 2 are aquatic, viz., *Nitrosomonas* (rod shaped 1x1.5 μm) and *Nitrosococcus* (coccoid, 1.5-2 μm). The NO_2^- oxidizers convert NO_2^- to NO_3^- (nitratation) and all are aquatic, viz., *Nitrobacter* (pearl shaped rod, 0.7x1.5 μm) *Nitrococcus* (coccoid, 1.7 μm) and *Nitrospina* (rod shaped 0.35x3 μm). The activity of *Nitrosomonas* and *Nitrobacter* was reported to be affected by light (Olson, 1981) with *Nitrobacter* being the most sensitive (Bock, 1965). They are also highly sensitive to sudden changes in temperature, pH below 6, reduction in available nutrients and several chemicals used for treating diseases in aquatic ecosystems (Burrows and Combs, 1968; Scott and Gillespie, 1972; Collins *et al.*, 1975; Spotte, 1979; Smith *et al.*, 1981; Bower and Turner, 1982). Nitrification, denitrification and nitrogen fixation are threatened also by contaminants such as heavy metals (Bouwman and Bloem, 2000).

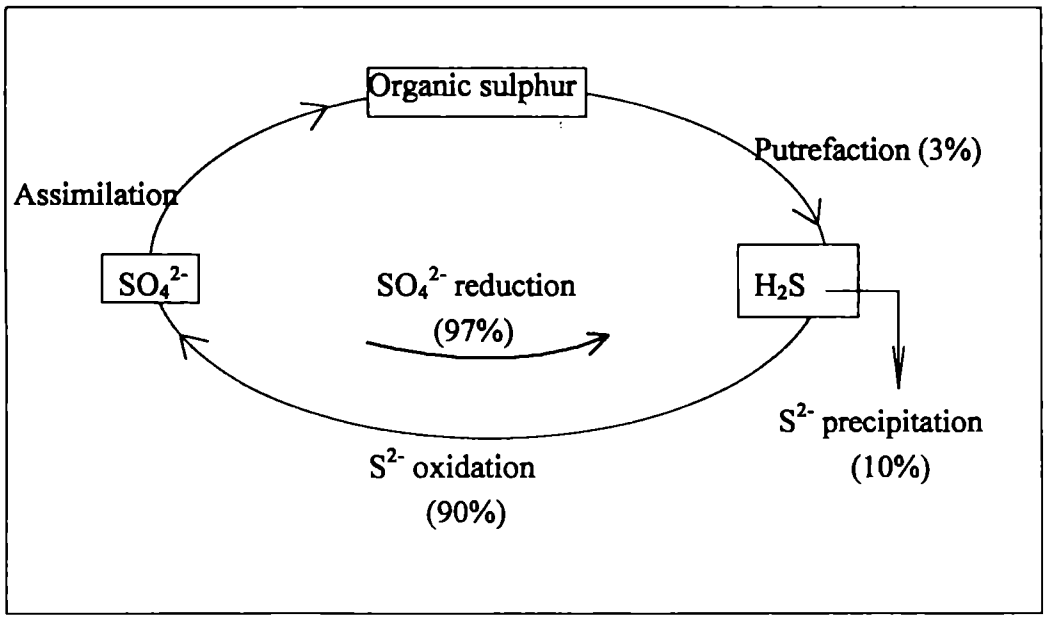
$$55\ NH_4^+ + 5\ CO_2 + 76\ O_2 \xrightarrow[NH_3\text{-monooxygenase}]{\textit{Nitrosomonas}\ \text{sp}} C_5H_7O_2N^- + 54\ NO_2^- + 52\ H_2O + 109\ H^+$$

$$400\ NO_2^- + 5\ CO_2 + NH_4^+ + 195\ O_2 + 2\ H_2O \xrightarrow{\textit{Nitrobacter}\ \text{sp}} C_5H_7O_2N^- + 400\ NO_3^- + H^+$$

The generation times of the autotrophic nitrifying bacteria are in the range of 10-30 h. Engel (1958) summarized that heterotrophs are also able to carry out nitrification, but to a lesser extent. The co-culture of a heterotroph *Arthrobacter* sp increased the nitrifying activity of an autotrophic *Nitrosomonas* strain possibly by reducing its lag phase (Kuenen and Gottschall, 1982; Kaplan, 1983). Bianchi *et al.* (1992) reported the ability of pseudomonads and asporogenous gram-positive rods isolated from an enclosed shrimp rearing facility to utilize NH_3. Joye and Hollibaugh (1995) reported that nitrification was rapidly and substantially reduced when 60-100 μm hydrogen sulphide (H_2S) was added to sediment slurries.



Nitrogen cycle



Sulphur cycle

Denitrification involves reduction of NO_3 to NH_3 free N_2. Bacteria capable of denitrification are predominantly facultative anaerobes. Jetter and Ingraham (1981) listed 73 genera capable of denitrification including common aquatic heterotrophs, viz., *Pseudomonas, Vibrio* and *Alcaligenes*. Denitrification rates are highest in early summer and freshly anaerobic water (Nedwell, 1984). Considering denitrification to be a two step process with methanol as C and energy source, the following reactions can be written

$$CH_3OH + 3NO^-_3 \longrightarrow 3NO^-_2 + CO_2 + H_2O$$

$$2NO^-_2 + CH_3OH \longrightarrow N_2 + CO_2 + H_2O + 2OH^-$$

The optimum pH for denitrifying bacteria lies between 7 and 8. They are sensitive to sudden changes in temperature. Most of the N_2 cycle process occur simultaneously in aquatic ecosystem. A well balanced microbial load would help in efficient cycling of N_2 in environment.

2.3.2. Sulphur cycle

Sulphur is one of the most abundant elements in our planet, present at approximately 520 mg/l level in the earth's crust (Goldschmidt, 1954). Sulphur is assimilated by many microorganisms and is the second most abundant anion in sea water (Austin, 1988).

Sulphate is one of the most common forms of sulphur found in habitats. In marine sediments, sulphate (SO_4^{2-}) and H_2S are constantly recycled between oxidation and reduction steps, predominantly carried out by two main groups of bacteria, viz., SO_4^{2-} reducers and sulphide (S^{2-}) oxidizers. Sulphate (SO_4^{2-}) is assimilated by bacteria and primary producers when they grow and incorporated mainly into sulphur containing amino acids of proteins. A variety of putrefying bacteria belonging to the genera *Proteus, Mycobacterium, Chromobacter, Bacillus, Micrococcus, Flavobacterium* and *Vibrio* produce H_2S by degrading the sulphur containing amino acids (Wetzel, 1983).

The H_2S is also produced directly from SO_4^{2-} by sulphate reducing bacteria (SRB) (Fry, 1987). These bacteria are all strict anaerobes and use SO_4^{2-} as terminal electron acceptor to oxidize organic compounds. The SRB in marine sediments have been reported to mineralize 25 - 50% of C. These include bacteria of the genera *Desulfovibrio,* (rod, curved shaped or spiral), *Desulfotomaculum* (spore forming rod) and *Desulfococcus* (coccoid). The primary habitat of SRB is the sediment with redox potential of –100 mV or below and that SRB are active within detrital particles of 100 µm thickness (Jorgensen, 1977a, b). Sulphate (SO_4^{2-}) reduction was reported to be highest in summer and lowest in winter (Fry, 1987). Suplee and Cotner (1996) found that new ponds initially had lower levels of SRB than old ponds, but the difference was lost by 17th week of grow out.

Once formed, the H_2S is either reoxidized to SO_4^{2-} or precipitated with iron to form insoluble ferrous sulphide. Reoxidation of S^{2-} is carried out biologically by a wide range of sulphide oxidizing bacteria (SOB). The H_2S oxidizers mainly include two groups of bacteria, viz., colourless sulphur bacteria and photosynthetic bacteria.

The colourless sulphur bacteria are all aerobic or microaerophilic and oxidize H_2S to S, which they store as S globules within their cells and they can use this S later to obtain energy when H_2S is unavailable (Austin, 1988). They include bacteria of the genera *Macromonas* (rod or bean shaped, 9x20 µm), *Thiovulum* (ovoid, 20 µm), *Thiospira* (spiral 2x50 –10 µm), *Thiobacterium* (nonmotile rod, 1x2 µm), *Beggiatoa* (filaments, 1-55 µm), *Thioplaca* (sheathed, 1-55 µm) and *Achromatium* (ovoid, 30-50 µm). There are a second group of colourless sulphur bacteria that oxidize H_2S and other inorganic sulphur to produce energy and form SO_4^{2-} but, there was no intracellular S deposition in these bacteria. They include bacteria of the genera *Thiobacillus* (rod, 0.5x1-4 µm), *Thiomicrospira* (spiral, 0.2-0.3 x 1-2 µm), *Thiosphaera* (coccoid), *Thiodendron* (vibrioid, 0.15-0.25 µm) and *Acidiphilium* (rod, 0.3-1.2 x 0.6-4.2 µm). *Thiobacillus denitrificans* can grow in

anaerobic conditions by converting NO_3^- to N_2. There are again a third group of colourless sulphur bacteria that require optimum temperature above 55°C for growth and, thus, are of lesser importance in S cycle. They include members belonging to genera *Thermothrix* (rod), *Sulfolobus* (spherical) and *Acidianus* (spherical) (Fry, 1987).

Under strict anaerobic conditions and in the presence of light, photoautotrophic bacteria of the family chlorobiaceae [green sulphur bacteria, e.g. i) *Chlorobium* -a non motile rod of 0.3-1.1x0.7-2.7 μm and ii) *Pelodictyon* – a rod of 0.8x1.8 μm] and Chromatiaceae [purple sulphur bacteria, e.g. i) *Chromatium* – an avoid rod of 1-6 x 2-15μm and ii) *Thiopedia* – a non motile rod of 1.5 x 2 μm] oxidize H_2S efficiently to fix CO_2 phototrophically (Fry, 1987). The green sulphur bacteria grow at lowest light intensities, cannot tolerate O_2 but can tolerate high H_2S concentrations. The purple sulphur bacteria need more light, are O_2 tolerant and H_2S sensitive, they always grow in a thin band just above the green sulphur bacteria and even may penetrate the oxygenated part of the H_2S /O_2 interface. Phototrophic bacteria are also found in sediments where light penetration and H_2S accumulation meet.

2.3.3. Carbon cycle

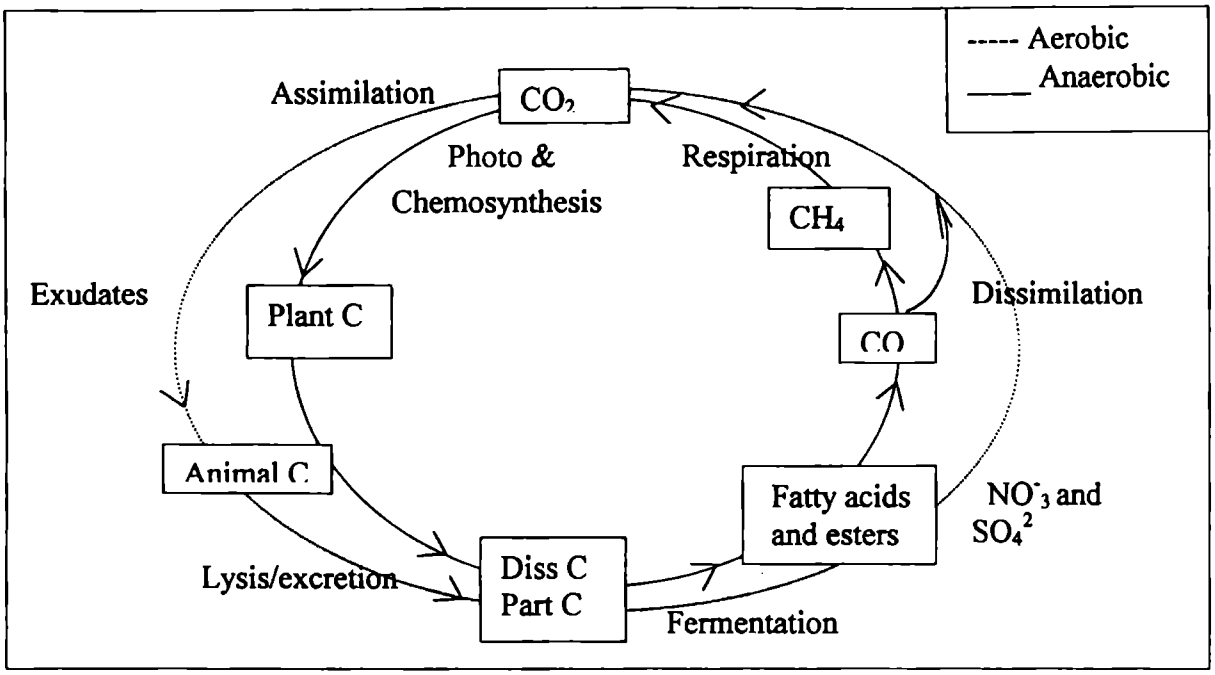
Carbon, one of the major constituents of all organic matter, undergoes recycling in nature, at the center of which stands CO_2. The earth's atmosphere contains about 0.032% (2.3 x 10^2 tons) by volume of CO_2 (Rheinheimer, 1992), but in sea water 50 times this amount is in solution. The cycle is very complex in water because many organisms are involved and many pools of different carbon compounds can be envisaged.

The C-cycle can be divided into assimilation, i.e., synthesis and transformation of organic material into multitude of natural C compounds and dissimilation which is the stepwise breakdown of all these substances by respiration by heterotrophic plants and animals (Rheinheimer, 1992).

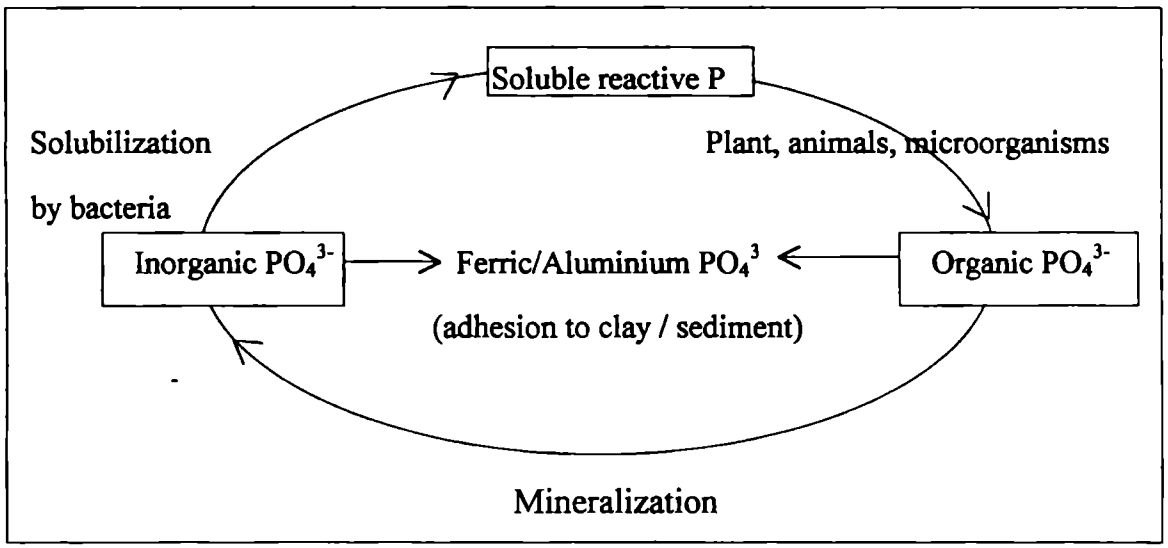
The C-fixing bacteria including cyanobacteria, photo and chemo autotrophic bacteria, etc. synthesize organic matter by fixing CO_2 and using light and other chemical substances such as NH_3, NO_2, NO^-_3 and S as their energy source (Fry, 1987). Some heterotrophs are also able to fix CO_2 in the dark and some predominantly autotrophic bacteria can use organic compounds as source of energy and C, these types are often called mixotrophs (Fry, 1987).

The decomposition of primary producers, when they die by microorganisms contributed to both the dissolved organic and particulate organic carbon compound pools. Heterotrophic bacteria grow on the particulate organic carbon and secrete exoenzymes that decompose it and the decomposition products enter the dissolved organic carbon pools. Most of the dissolved organic carbon is respired by heterotrophic microorganisms to CO_2. The wide range of heterotrophic bacteria involved in the entire process from decomposition of primary producers to production of CO_2 mainly belong to the genera of *Flavobacterium, Pseudomonas, Vibrio, Aeromonas* and *Alcaligenes.* Pike (1975) reported that 90-95% of bacteria in oxidation ponds are *Pseudomonas, Achromobacter* and *Flavobacterium,* thus demonstrating their predominance in these systems. The SRB and denitrifying bacteria are also known to mineralize carbon. The zone near the surface containing the redoxycline, is often the site of most bacterial activity when gross measures are used (Fry, 1987).

A portion of the dissolved organic carbon will be converted to methane (CH_4), probably mainly through acetate by methanogens. The CH_4 producing bacteria are morphologically diverse but physiologically similar group and most of the 7 genera are rod shaped (*Methanobacterium*) or coccoid (*Methanogenium*) but one genus (*Methanospirillum*) have spirally shaped members (Fry, 1987). All are anaerobes and grow best at redox potential of – 200 mV or below. However, their growth is limited by SRB as they complete with them for acetate and hydrogen (Nedwell, 1982; 1984).



Carbon cycle



Phosphorus cycle

The CH_4 produced in sediments by methanogens is not oxidized anaerobically but rises into the water column and once it reaches the oxygenated layer is rapidly oxidized by methanotrophic bacteria, *Methylomonas, Methylococcus* and *Methylosinus* (Fry, 1987). They are microaerophilic and use CH_4 as carbon and energy source to produce CO_2 (Capppenberg, 1972; Rudd and Hamilton, 1975).

2.3.4. Phosphorus cycle

Phosphate (PO_4^{3-}) is one of the most important limiting factors for plant life in many waters. Phosphorus particularly as a constituent of nucleic acids, and a vital element for all organisms, is also present in phospholipids, phosphorylated sugars, phytin, ATP etc.

Phosphorus cycle involves conversion of inorganic phosphorus to organic form and vice versa. Phosphorus is taken up by plants as pyrophosphates, that is changed to organic P compounds and from these, PO_4^{3-} are released mainly due to the action of microorganisms (Rheinheimer, 1992). During cycling P may get immobilized due to adhesion to clay particles or formation of ferric or aluminium phosphates.

Solubilization of inorganic phosphates is carried out by a wide range of microorganisms, viz., *Pseudomonas, Achromobacter, Flavobacterium, Arthrobacter, Streptomyces* and *Aspergillus* (Botto, 1988). This solubilized PO_4^{3-} is taken up by phytoplankton and plant for production of organic substances. Mineralization of organic P is carried out by a variety of microorganisms such as *Arthrobacter, Proteus, Serratia, Streptomyces, Aspergillus* and *Rhizopus* (Botto, 1988).

Although many organisms have the ability to hydrolyze phytate *in vitro*, this form of organic PO_4^{3-} has a very strong affinity for adsorption on clay particles, which prevent accesses by the phytases produced by the organisms.

Consequently, phytate tends to accumulate and is the major form of organic phosphorus found in most soils (Botto, 1988).

2.4. Bioremediation: A new concept

Bioremediation has almost become a household word these days, implying the use of biological agents to control problems of environmental pollution. A prominent example of bioremediation, often cited in popular press and scientific magazines, is that of Exxon Valdez Oil Spill, where indigenous microorganisms were supplied with an oleophilic fertilizer and allowed to proliferate and consume the spilled oil (Chakrabarty, 1992).

Bioremediation is defined as the process by which microorganisms are stimulated to rapidly degrade hazardous organic contaminants to environmentally safe levels in soils, subsurface materials, water, sludges and residues (Thomas *et al.,* 1992). Stimulation is achieved by the addition of nutrients and a terminal electron acceptor usually O_2, because most biological reactions occur faster under aerobic than anaerobic conditions. Under anaerobic conditions, NO^{3-} has been used as the terminal electron acceptor.

Bioremediation is a pollution treatment technology that uses biological systems to catalyze the destruction or transformation of various chemicals to less harmful forms (Atlas and Unterman, 1999). Bioremediation is cost effective, environmentally sound and increasingly the preferred choice of remedial technology for clean up operation.

The objective of bioremediation programme is to immobilize contaminants (reactants) or to transform them to chemical products no longer hazardous to human health and environment. The end products of effective bioremediation are non-toxic and can be accommodated without harm to the environment and living organisms (Atlas and Unterman, 1999).

The selection of most effective bioremediation strategy is based on - a) characteristic of the contaminants (toxicity, molecular structure, volatility, solubility and susceptible to microbial attack) b) the contaminated site (hydrology, geology, soil type and climate and the legal, economic and political pressures felt by the site owner and c) the microbial process that will be exploited, such as pure culture, mixed culture, their respective growth conditions and supplements.

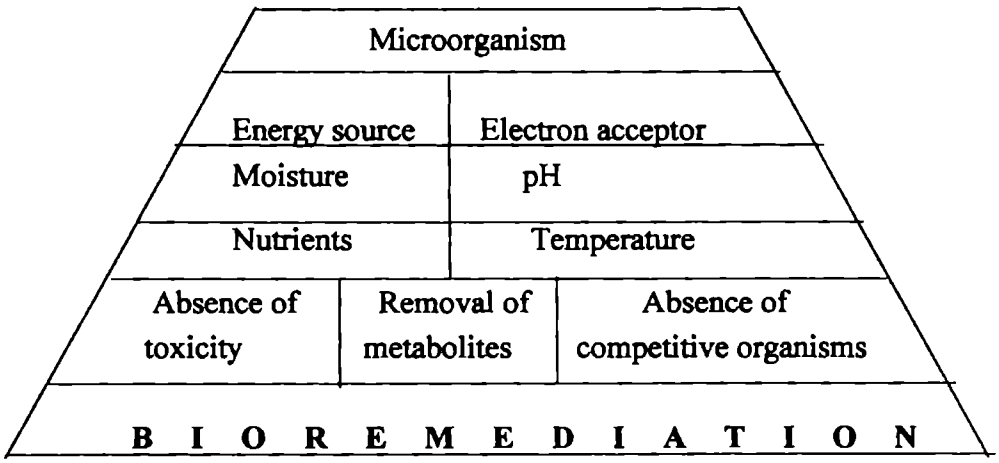
The general approaches to bioremediation are – (1) Intrinsic bioremediation – Intrinsic bioremediation is the management of contaminant biodegradation without taking any engineering steps to enhance the process. It uses the innate capabilities of naturally occurring microbial communities to metabolize environmental pollutants. Because intrinsic bioremediation occurs in the landscape where both indigenous microorganisms and contaminants reside, this type of bioremediation necessarily occurs *in situ*. It may be used along or in conjunction to other remediation techniques. For intrinsic bioremediation to be effective, the rate of contaminant destruction must be faster than the rate of contaminant migration. 2) Engineered bioremediation – Engineered bioremediation either accelerates intrinsic bioremediation or replaces it completely through the use of site modification procedures that allow concentration of nutrients, electron acceptors, or other materials to be managed in a manner that hastens biodegradation reactions. Engineered bioremediation may be chosen over intrinsic bioremediation because of considerations of time, cost and liability. It falls into 2 categories – (a) Biostimulation refers to the addition of specific nutrients to a waste situation with the hope that the correct, naturally occurring microbes are present in sufficient numbers and types to breakdown the waste effectively. This assumes that every organisms needed to accomplish the desired treatment results is present. (b) Bioaugmentation involves the addition of specifically formulated microorganisms to a waste situation. It allows one to control the nature of the biomass. It ensures that the proper team of microbes is present in the waste situation in sufficient type, number and compatibility to attack the waste

constituents effectively and break them down into their most basic compounds (Burlage *et al.*, 1999)

Biodegradation of naturally occurring and synthetic organic compounds requires or is faster when several species of microorganisms are present. In instances where the indigenous microflora fails to degrade the target compounds or has been decimated by the presence of toxicants, microorganisms with specialized metabolic capabilities may be added (Thomas *et al.*, 1992).

In bioremediation, the emphasis, so far, has however, been on the use of natural microorganisms rather than genetically manipulated ones, because of the adverse public perception on the release of genetically engineered microorganisms as well as various regulatory constraints on their use (Chakrabarty, 1992). The major reason for using genetic selection in the decontamination of polluted environment is the fact, that in many cases, natural microorganisms have not evolved the genetic competence to utilize a synthetic compound. To generate new degradative capability against a newly made synthetic compound, a microorganism must evolve the appropriate genes encoding enzymes that would have high affinity for the target chemical or its intermediate products as substrates.

The requirements for an effective bioremediation is illustrated (Cookson, 1995) pyramidically as follows.



Bioremediation has some definite advantages over other treatment technologies in that it can be done at site, facilitates permanent elimination of waste, biological systems are cheaper, evokes positive public acceptance, minimum site disruption, eliminates transport cost and liability and can be coupled with other treatment techniques.

The bioremediation microorganisms frequently identified as active members of microbial consortium are *Alcaligenes denitrificans, Arthrobacter globiforms, Arthrobacter* sp, *Bacillus* sp, *B. megaterium, Flavobacterium* sp, *Mycobacterium, M. vaccae,* Methanobacteriaceae, *Nitrosomonas europaea, Nocardia* sp, *N. corallina, N. erythropolis, Pseudomonas* sp, *P. aeruginosa, P. putida, P. cepacia, P. fluorescens,P. glatheri, P. mendocina, P. methanica, P. paucimobilis, P. testosteroni* and *P. vesicularis* (Baker and Hersan, 1994).

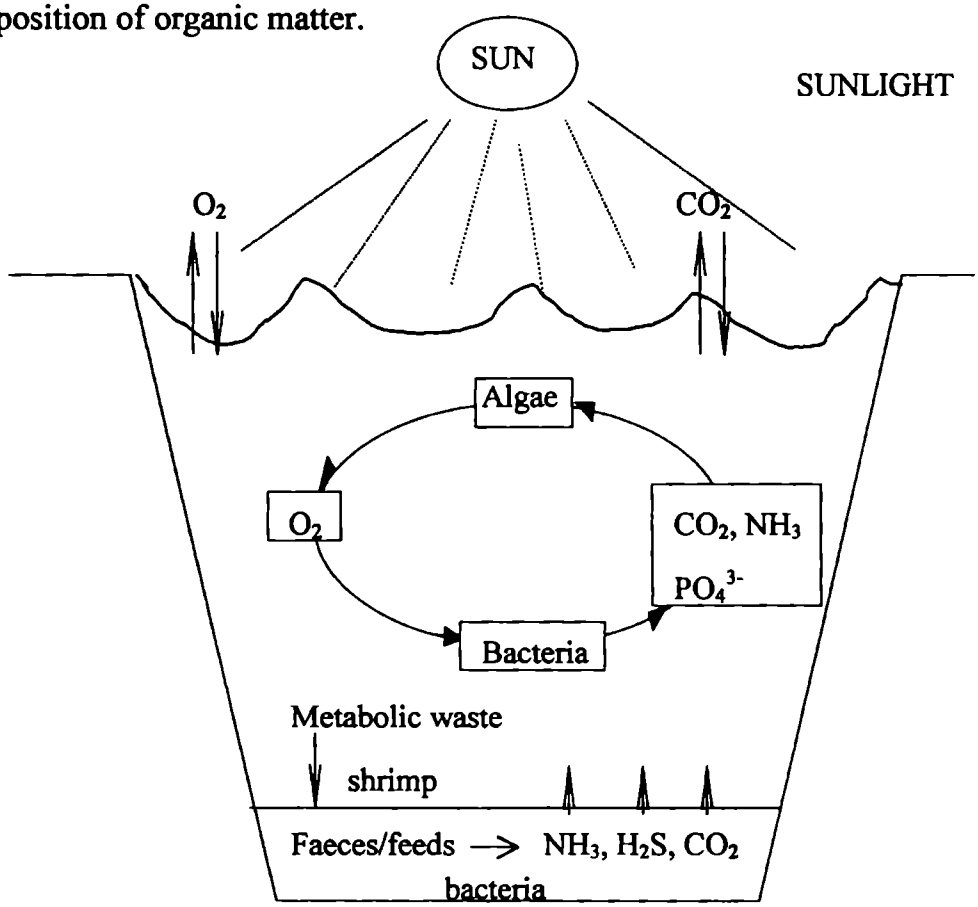
The spectrum of compounds susceptible to bioremediation is naturally occurring, have simpler molecular structure, are non-toxic and serve as a growth substrate for aerobic microorganisms. A few examples of such compounds are inorganic ones as NO^-_3, SO_4^{2-}, PO_4^{3-} and NH^+_4 compounds and petroleum hydrocarbons (xylene, toluene, benzene, ethylbenzene, alcohols and ketones). In contrast, compounds that are resistant to microbial metabolism, have complex molecular structure, low water solubility, strong sorptive interactions, toxic and do not support the growth of microorganisms. A few example of such compounds are halogenated aliphatic and aromatic compounds (Burlage *et al.,* 1999).

2.5. Bioremediation in shrimp aquaculture

2.5.1. The need/necessity

It is a golden rule that successful intensive shrimp culture requires intensive management to maintain good pond water quality. The pond water quality changes quickly because of the input of large quantities of high quality feeds. Most of these feeds eaten by shrimps are eventually excreted as metabolic wastes that add

inorganic nutrients and organic matter to the bottom of ponds. According to Briggs and Funge-Smith (1994) only 21% of nitrogen and 13% of phosphorus of the feed input (at a conversion rate of 2) gets incorporated into flesh of shrimp. On the other hand, Primavera (1994) has reported only 17% incorporation of feed input by shrimp. The ponds, thus, become eutrophic with active decay and assimilation of left over feed and metabolic wastes carried out by microorganisms. As a result of microbial activity under aerobic conditions the organic matter is converted to inorganic compounds such as PO_4^{3-}, NH_3 and CO_2. The microbial process of converting organic matter to inorganic compounds is called mineralization. Some of these organic compounds serve as nutrients to stimulate algal growth, which in turn produce oxygen, which the microorganisms need for decomposition of organic matter.



Role of microorganisms in the metabolic cycle in shrimp ponds (Anon, 1993).

Many a times the appropriate species of microorganism for purifying water/sediment and appropriate physico-chemical conditions may not be always present in the pond to promote rapid growth and speedy mineralization. The newest attempt being made to improve water quality in intensive shrimp culture is the application of bacteria 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 (Anon, 1993).

Beneficial, ecofriendly bacteria are a must for healthy prawn culture. Moreover, water treatment with chlorine, iodophores and antibiotics kill the beneficial autochthonous microbes as well as pathogenic allochthonous microbes, reducing the fertility of water. Microorganisms are known to play an important role in nutrient recycling in any aquatic environment (Rheinheimer, 1992). Water quality in aquaculture system is, to a large extent, controlled by microbial biodegradation of organic residues (Avnimelech *et al.,* 1995). Therefore, attempts are being made to improve water quality in intensive shrimp culture ponds through application of bacterial population capable of degrading organic matter in the ponds.

Many scientists feel that addition of bacterial seed stock is pointless. Their beliefs are based on misinterpretation of ubiquity principle. The principle of ubiquity states that bacteria may be found anywhere, it does not state that all bacteria are found everywhere all the time. The underlying assumptions of the following misconceptions are not true-(1) that the appropriate species for water purification and organic sediment decomposition are always present (2) that appropriate physico-chemical conditions are always present to permit rapid growth and (3) that bacterial growth is not limited by process such as predation (Ehrlich *et al.,* 1988). Bird and Kalff (1984) reported that addition of specific microbial

mixture or manipulation of the microflora of the deteriorated environment may hasten the process of mineralization, thereby bringing about rapid purification.

The practice of bioremediation (bioaugmentation) is applied in shrimp culture, but success varies greatly, depending on the nature of the products used and the technical information available to the end user. The bacteria that are added must be selected for specific functions that are amenable to bioremediation, and added at high enough population density, and under the right environmental conditions. Bioaugmentation is a significant management tool, but its efficacy depends on understanding the nature of competition between species or strains of bacteria.

2.5.2. Application of bioremedial products in shrimp aquaculture

There are many reports on the success as well as unsuccessful results of using bacterial products in aquaculture.

In the 1980's Alken-Murray Corporation changed the economic feasibility of bacterial treatment by offering highly concentrated, non-pathogenic and cost effective Alken-Clear Flo ® formulae to degrade excess nutrients, chemical pollutants and NH_3 in traditionally high volume shrimp producing pond waters. Alken-Clear Flo ® 1000 and 1002 include basic spore forming, waste degrading strains of *Bacillus* to reduce organic loadings in the water column, preventing a built of sludge on pond bottom by 60%. Alken-Clear Flo ® 1100, 1200 and 1400 contain *Nitrosomonas europaea*, which degrade NH_3 to NO_2^- and *Nitrobacter winogradskyi*, which degrade NO_2^- to NO_3^- in the aerobic environment of the water column and to N_2 gas in the anaerobic environment of the bottom sludge or gravel (www.alken-murray.com).

Boyd *et al.* (1984) studied the effect of commercial bacterial suspension (AQUA. BACTA. AID) and found that it did not have any significant effect on water quality parameters such as total ammoniacal nitrogen (TAN) concentration,

NO_2-N concentration, NO_3-N concentration, total phosphorus concentration, biological oxygen demand (BOD) and chemical oxygen demand (COD). On the other hand, Ehrlich *et al.* (1988) reported positive effects of the same bacterial consortium in its ability to accelerate nitrification, increase decomposition of organic solids (10-12 cm/month), reduce excessive algal growth, facilitate oxygenation and aid in transformation of agricultural wastes into faunal biomass.

Sanjiban Microactive is a liquid stimulator developed from complex fermentation process. It is an organic extract enriched with natural enzymes that activates and rapidly multiplies healthy organisms already present in the effluent system. In shrimp ecopond system under warm climatic conditions, the typical removal efficiency of various pollutants that have been achieved are: total suspended solids (TSS) :- 80-95%, BOD :- 85-98%, COD:- 80-93%, NH_4-N :- 85-95%, phosphorus :- 90-95% and *E. coli* :- 99%. Furthermore, production of 500-1000 kg shrimp/ha and 5000-6000 kg fish/ha can also be harvested (www.supplyindia.com/microactive. htm).

Porubcan (1991a; b) reported on two attempts at bacterial treatments to improve water quality and production yield of *Penaeus monodon* – (1) floating biofilters pre-inoculated with nitrifying bacteria decreased the amounts of NH_3 and NO_2^- in the rearing water. This treatment also increased shrimp survival (Porubcan, 1991a) and (2) the introduction of *Bacillus* sp in close proximity to pond aerators reduced COD and increased shrimp harvest (Porubcan, 1991b).

Chiayvareesajja and Boyd (1993) studied the effect of a bacterial product (ACCELOBAC) on TAN concentration and reported that treatment of pond water with up to 40 mg/l of ACCELOBAC caused no change in TAN concentration over a 10 day period. Tucker and Lloyd (1985) found no benefits of bacterial augmentation in lowering TAN concentration or improving any other aspects of water quality.

An intensive shrimp culture pond in Thailand which use a commercial bacterial product throughout the culture period got good production of 6806 Kg/ha (FCR 1.4 and survivality 80%), demonstrating the possibility of using bacterial products to maintain good water quality (Anon, 1993). The use of 'EPICIN' a commercial bacterial product of EPICORE Network in shrimp ponds in Indonesia has produced the largest harvest ever recorded by the ponds involved and profits were up to 5 times greater than from ponds not treated. The major and probably most significant effect of 'EPICIN' on water quality was in its ability to reduce NH_3 concentration. Following 'EPICIN' application, concentration of other nutrients including NO_2^-, NO_3^- and H_2S were also reduced to well below than that in untreated ponds (Anon, 1995). Funge-Smith and Hawthorn (1996) tested 5 commercially available bacterial products for their efficacy in improving water quality under laboratory conditions. They reported that none of the products had significant effect on TAN as well as NO_2^-N concentrations.

Shrimp farms in Indonesia that use the Detritus Management System (DMS) – range of *Bacillus*, do not have problems from diseases caused by luminescent *Vibrio* sp (Moriarty, 1996). Chandrika (1999) reported on bioaugmentation with 10^9/g of DMS – *Bacillus* to mineralize and reduce the faecal matter of shrimps and left over feed in intensive aquaculture. Anon (1999) studied the effect of a bioaugmentor, viz., Bioklean MX – 1 (bacterial product) for removal of toxic NH_3 from shrimp culture systems and reported that Bioklean @ 12 ppm was effective in reducing the concentration of NH_3. They also studied the efficacy of *Pseudomonas* ($1x10^6$/ml) on removal of NO_2^- from shrimp culture systems and reported that 5 ml/l of *Pseudomonas* was effective in decreasing NO_2^- concentration. They further studied effect of plant by-products and extracts on removal of NH_3 from shrimp culture system. According to them, neem seed oil @ 100 ppm, neem leaf extract @ 90 ppm and custard apple seed oil @ 90 ppm were all effective in reducing NH_3 concentration.

In China, Li Zhuojio *et al.* (1997) reported on the application of a mixture of several strains of photosynthetic bacteria (*Rhodomonas* sp) to improve the shrimp culture water and have achieved remarkable results. There was a total elimination of NH_3-N, H_2S and organic acids coupled with improvement in water quality and balancing of pH resulting in increase in body length and weight of shrimps. They concluded that the bacterial population might have chemical actions such as oxidation, nitrification, ammonification, denitrification, N_2 – fixation and sulphurication.

An alternative way to maintain high water quality in intensive shrimp culture is biological treatment based on the use of filters with a high surface / volume ratio, pre-colonized by microorganisms that absorb excess nutrients from the water. A biological filter for filtration of shrimp culture water has been developed recently by Bioworld. The filter occupying a volume of 11% of water volume under production, provides a large surface area (20 m^2) for many biological processes: ammonification, nitrification and denitrification (www.biogroup.gzea.com). The bioremedial products offered by New China Limited, are very useful to those raising shrimp in ponds. They create larger, more healthy shrimps and lessen mortality by avoiding NH_3 build up; thus increasing profits (www.generic.com). Bacta Clean – ALGAE, Type 2 is a bioremedial product used for shrimp aquaculture pond maintenance as it prevents NH_3 build up, slime formation and algal growth. Moreover, it reduces NO_3^- added to pond water by shrimp faeces and scavenges bottom sludge materials (www.regenesis.com).

Prabhu *et al.* (1999) studied the effect of a commercially available probiotic (NS series Super SPO) on the water quality parameters of 4 ponds in a shrimp farm. The product was soaked in pond water @ 1g/200ml and activated by vigorous aeration for 4 h. After activation, the liquid containing the slurry was sprinkled uniformly over the surface water in each pond. The results of the

experiment showed a marked decrease of NH_4-N concentration in the 3 experimental ponds with progressive days of culture (DOC); while there was a marked increase in NH_4-N concentration in the control pond. The total heterotrophic bacterial count increased by 10^4 (from 10^3 to 10^7) cfu/ml in water and by 10^5 (from 10^3 to 10^8) cfu/g in sediment in the control pond, which is much more when compared to the increase by 10^3 (from 10^3 to 10^6) cfu/ml in water and 10^3 (from 10^4 to 10^7) cfu/g in sediment of experimental tanks.

Recently, Oppenheimer Biotechnology is co-operating with the Philippines Company Envirogenics, Inc. to evaluate the use of Oppenheimer Formula 1 product to enhance production in shrimp pond culture. The application of Oppenheimer Formula 1 to sediments and water of 2-5 acre ponds have been shown to double the normal production in the same time period. There is also evidence that microbes may reduce the mortality caused by other competing microorganisms, control algae, decrease BOD and COD, decrease NH_3, NO_2^- and NO_3^- (www.obio.com©2001 Oppenheimer Biotechnology, Inc.).

Shan and Obbard (2001) from the Department of Chemical and Environmental Engineering, National University of Singapore isolated cultures of nitrifying bacteria from intensive prawn aquaculture water and enriched them using continuous and batch enrichment techniques. Cultures were immobilized on to porous clay pellets to enhance cell density and when applied to water with high TAN concentrations have been found to exhibit high TAN removal rates.

Moriarty (1996) has summarized the reasons for inefficiency of few bacterial mixtures under field conditions. His contention is that the bacterial products might have lacked the sufficient number of right strains of bacteria to be effective or it is possible that the bacteria were not viable. It is apparent that many suppliers of bacterial products are unaware of the physiological and ecological requirements of their bacteria. For example, some contain purple sulphur bacteria that will remove S_2^- only when conditions are anaerobic and light is present.

Nitrifying bacteria are autotrophic and need CO_2 as their carbon source and oxidize NH_3 for their energy. They are very difficult to maintain, require oxygen and are slow growers. If these conditions are not provided, the activity of these bacteria will be inhibited.

The use of macroorganisms as effective bioremediators in shrimp culture system has led to the development of new culture models such as shrimp-shellfish (molluscs/oysters), shrimp-fish and shrimp-algae. The seaweed *Gracilaria* is an attractive species to be grown as part of polyculture with molluscs in a biological treatment system because it can remove soluble nutrients, nitrogen and phosphorus, which are not absorbed by molluscs. The culture of shrimp with fish (especially predatory fish such as *Fugu* sp, perch and tilapia) is found to be the most successful for preventing disease occurrence. It is believed that predatory fish may eat sick or morbid shrimps, thereby eliminating the spread of diseases in shrimp culture pond. Mangroves have also been suggested to treat shrimp pond effluents in that it acts as biological filters by trapping pollutants, i.e., excess nutrients, suspended solids, heavy metals, toxic hydrocarbons, etc. (Babu *et al.*, 1998).

Bioremediation and its efficacy are debatable topics. This may have potential applications in aquaculture. A thorough and detailed investigation is, however, necessary to understand the behaviour as well as environmental requirements of beneficial microbes that exist in shrimp ponds. Large scale laboratory and field studies are required to clearly demonstrate the ability of the microbes as bioremediators. Viability and economics are the other vital aspects, which have to be considered before adopting these methods.

Chapter - 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Bacteriological media and diluents

The most common bacteriological media used in this study include nutrient agar (NA), Winogradsky medium, Sulphur oxidizing bacterial medium, Sulphate reducing bacterial medium, Sea water nitrate broth, Trypticase soy agar (TSA) and Trypticase soy broth (TSB). Dehydrated media such as NA, TSA and TSB were procured from Hi-Media, Mumbai (India). The pH of the medium was adjusted to the required level using 1M sodium hydroxide solution and / or 1M hydrochloric acid before sterilization. All media had a minimum of 1% (w/v) sodium chloride (NaCl) unless otherwise specified. One % NaCl (w/v) solution was used as diluent for the enumeration of bacteria.

3.1.1. Sterilization

All glasswares were sterilized in an oven at 180°C for 1 h. Bacteriological media, diluents and autoclavable labwares were generally sterilized in an autoclave at 121°C for 15 min. unless otherwise specified. Polythene bags were sterilized by ultra-violet (U-V) rays for 30 min.

3.2. Selection of study area

The present study was initiated in October, 2000 and continued up to July, 2002. In West Bengal, shrimp farming is practiced in all the maritime districts, viz., 24 Parganas (North), 24 Parganas (South) and Midnapore (East). The district 24 Parganas, North and South have vast potential area of 29,026 ha and 10,386 ha, respectively for shrimp farming, where culture is being traditionally done in *"Bheries"*. The Midnapore (East) district has 3, 342 ha of potential areas suitable for shrimp farming (Upadhyay, 2001). Shrimp farming in this district is carried out

in dug-out ponds scientifically and the types of culture include improved traditional, stagnant pond culture with or without management, modified extensive and semi-intensive (Annexure 1). The dug out ponds of Midnapore (East) and *"Bheries"* of 24 Parganas (North and South) districts were selected for this study.

3.2.1. Description of the study area

The locations of the study area are depicted in Figs.1a – d. The detailed activities and the type of culture practices are presented in Table 1.

Table 1. Description of the study area

Area	Location		Type of culture	Major activity
	Latitude	Longitude		
Midnapore (East) district				
Kanthi zone	21°48' N	87°45'E		
Kalinagar			Modified extensive	Shrimp farming and agriculture
Auria			Stagnant pond Culture	Shrimp farming and agriculture
Soula			Semi-intensive	Shrimp farming and agriculture
24 Parganas (South) district				
Canning zone	22°20'S	88°40'E	Improved traditional	Shrimp farming and agriculture
Malancha zone	22°	88°45'E	Traditional	Shrimp farming and agriculture
24 Parganas (North) district				
Khariberia zone	22°25'N	88°35'E	Traditional	Shrimp farming and agriculture

Fig: 1a. A complete map of West Bengal.

Fig: 1b. A map showing the sampling sites of Midnapore district.

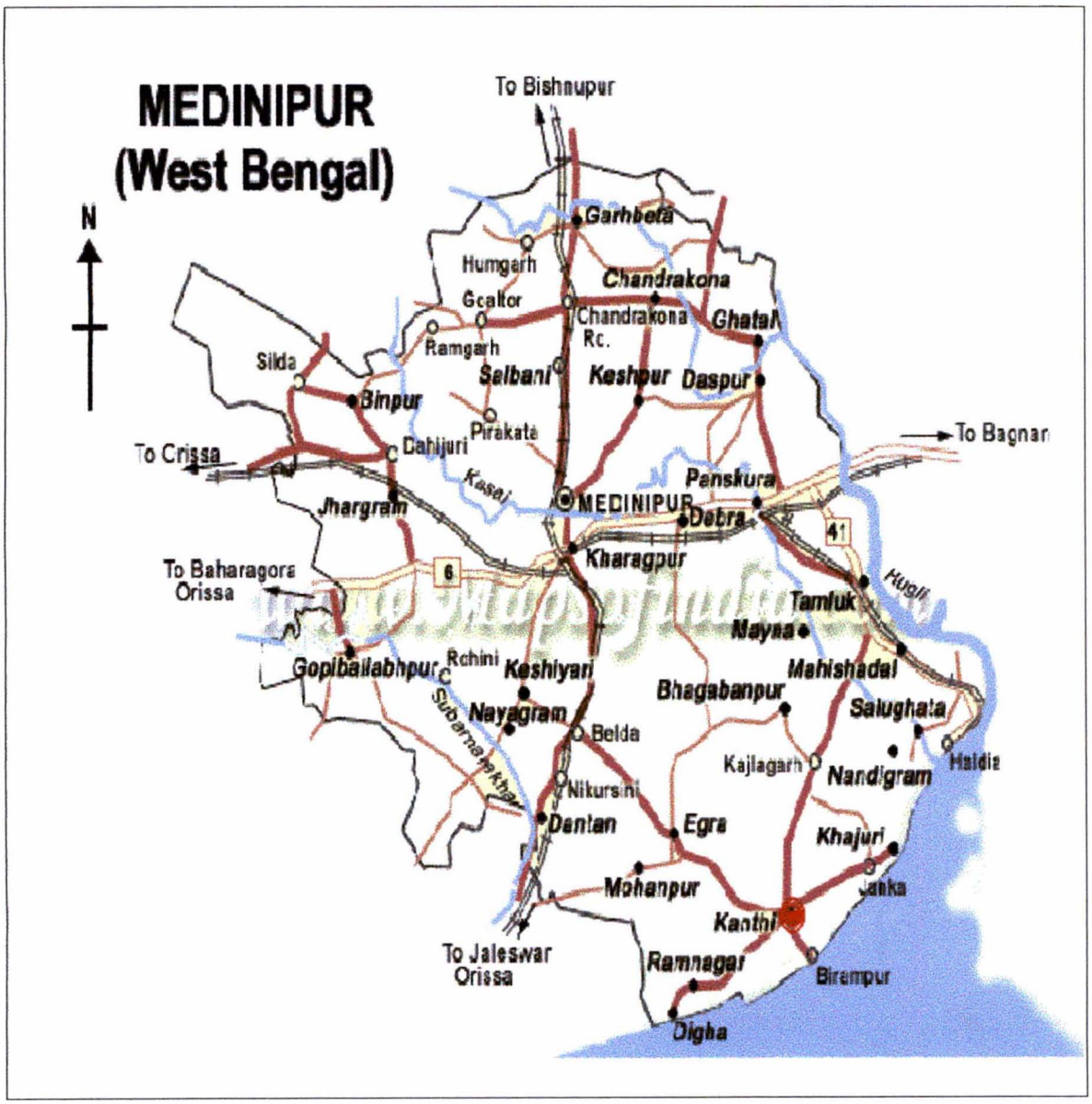


Fig: 1c. A map showing the sampling sites of South 24 Parganas district.

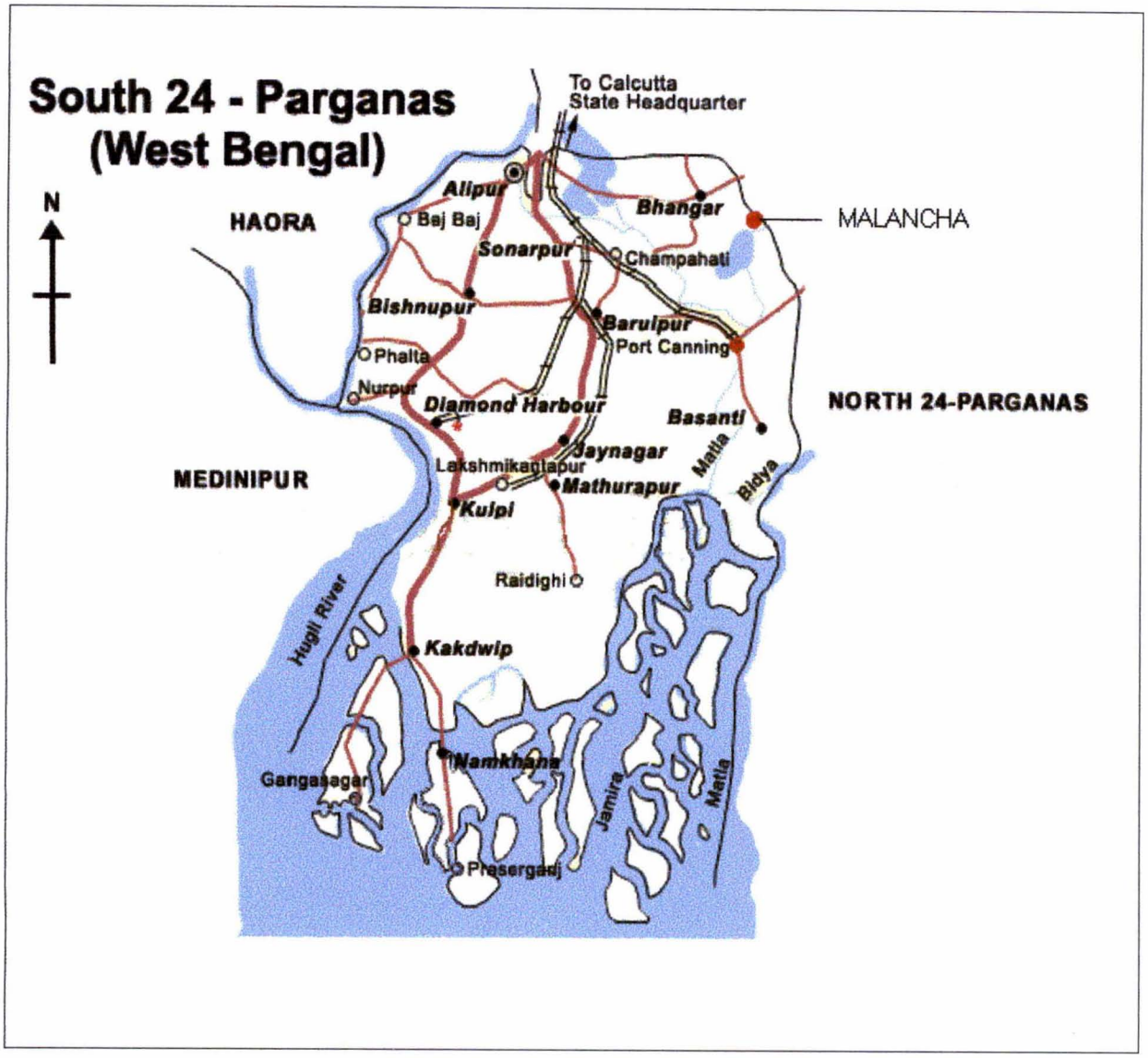


Fig : 1d. A map showing the sampling sites of North 24 -Parganas district.

3.3. Sampling procedure

3.3.1 Sample collection

Samples of shrimp grow-out pond water and pond sediment, from the culture systems of different zones as described in Table 1 were collected at regular intervals. Samples of pond water were collected in sterilized polypropylene bottles of 250 ml capacity. Pond sediment samples were collected at four places, viz., near the inlet, close to outlet, pond center and close to feeding tray in scientifically managed ponds and/or at four sides of the *Bheries* using sterilized plastic jars and transferred immediately to U-V sterilized polythene bags. All the samples were placed in insulated containers and brought to the laboratory within 4 h of collection or 24 h in case of outstation samples. Samples of pond water for physico-chemical analyses were also collected separately as above from each area.

3.4. Sample analysis

3.4.1 Physico-chemical analyses

The various physico-chemical parameters analysed in the present study include salinity, temperature, pH and nutrient concentrations (ammonia, nitrate and nitrite) for water and pH for sediment. The temperature and pH of water were measured by mercury thermometer and digital pH meter (Hanna, Portugal), respectively. The salinity was measured using a refractometer (Erma, Japan). The nutrient concentrations, viz., ammonia, nitrate and nitrite were measured by phenate, U-V spectrophotometric screening and colorimetric method respectively, (APHA/AWWA/WEF, 1998) using double beam U-V vis spectrophotometer (Techcomp 8500PC). The pH of sediment was measured using a soil pH meter.

3.4.2. Bacteriological analyses

3.4.2.1. Total heterotrophic counts

Spread plate technique was followed for total heterotrophic bacterial enumeration. Water samples collected in sterile bottles were suitably diluted (up to 10^{-3}) using 1% saline. Aliquots of 0.1 ml each of dilutions were spread on the surface of prepoured and dried NA plates. Inoculated plates were incubated at ambient temperature (30±2°C) for 48 h and the colonies counted.

Sediment samples collected in sterile plastic bags at four places from each pond, were pooled together and mixed thoroughly in a homogenizer before analysis. The homogenized samples were suitably diluted up to 10^{-5} using 1% saline and bacterial enumeration was done by spread plate technique as for water samples.

Nutrient Agar (Hi-Media, 1991)

Ingredients	
Peptic digest of animal tissue	5 g
Sodium chloride	10 g
Beef extract	1.5 g
Yeast extract	1.5 g
Agar	15 g
Distilled water	1000 ml
pH	7.4±0.2

Sterilization by autoclaving at 121°C for 15 min.

3.4.2.2. Nitrifying bacteria

3.4.2.2.1. *Nitrosomonas* sp

The most probable number (MPN) technique was used to enumerate the nitrifier, *Nitrosomonas* sp of water and sediment samples. The water and sediment samples were suitably diluted up to 10^{-3} and 10^{-5}, respectively using 1% saline and

inoculated into pre-sterilized modified Winogradsky medium (Rodina, 1972). Inoculated tubes were incubated for 6-15 days at ambient temperature (30±2°C). *Nitrosomonas* sp. oxidizes NH_3 to NO_2, so the presence of NO_2 in tubes indicates the presence of *Nitrosomonas* sp.

Detection of NO_2 in tubes was carried out using Greiss reagent. About 7-10 mg of dry reagent was taken with a spatula and placed in the depression of a clean porcelain plate and 3-4 drops of culture was added. Development of a brick red or pink colour immediately indicated the presence of NO_2.

Modified Winogradsky medium (Rodina, 1972)

Ingredients	
Ammonium sulphate	2 g
Dipotassium hydrogen phosphate	1 g
Sodium chloride	0.5 g
Magnesium sulphate. 7 hydrate	0.5 g
Manganese sulphate. 4 hydrate	Trace
Ferric sulphate. 9 hydrate	Trace
Distilled water	1000 ml
pH	8.5

Sterilization by autoclaving at 121°C for 15 min

Greiss reagent	
α - Naphthylamine	1 g
Sulphanilic acid	10 g
Tartaric acid	89 g

The above ingredients were mixed thoroughly, ground in a mortar to a fine powder and kept in a dark bottle with glass stopper.

3.4.2.2.2. *Nitrobacter* sp

The MPN technique was used to enumerate the nitrifier, *Nitrobacter* sp in sediment and water samples. The water and sediment samples were suitably

diluted up to 10^{-3} and 10^{-5}, respectively using 1% saline and inoculated into pre-sterilized Winogradsky medium (Rodina, 1972). All the tubes were incubated at room temperature (30±2°C) for 6-15 days. *Nitrobacter* sp oxidizes NO_2 to NO_3. The presence of *Nitrobacter* sp is noted by appearance of NO_3 in tube. Diphenylamine reagent was used for the detection of NO_3 in tubes.

A series of test tubes were thoroughly washed and dried. About 0.1 g of urea, followed by 7 ml of distilled water and 1 ml of culture were added to each test tube. Finally, 2 ml of 1:1 diluted sulphuric acid (H_2SO_4) with distilled water was added. The tubes containing this mixture were allowed to stand for 16-18 h. An aliquot of 1 ml of this mixture was taken in a test tube. One drop of 20% NaCl was added followed by 2 ml of 0.017% solution of diphenylamine in H_2SO_4 which was carefully deposited down the wall of the test tube. Presence of NO_3 was indicated by the development of blue ring at the border of the two liquids.

Winogradsky medium (Rodina, 1972)

Ingredients	
Sodium nitrite	1 g
Sodium carbonate	1 g
Sodium chloride	0.5 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulphate. 7 hydrate	0.3 g
Ferrous sulphate. 7 hydrate	0.4 g
Distilled water	1000 ml
pH	8.5

Sterilization by autoclaving at 121°C for 15 min.

Diphenylamine reagent

Diphenylamine	17 mg
Sulphuric acid (conc)	85 ml
Distilled water	15 ml

3.4.2.3. Sulphur oxidizing bacteria (SOB)

The SOB was enumerated following MPN technique. The water and sediment samples were suitably diluted up to 10^{-3} and 10^{-5}, respectively using 1% saline, inoculated into sterilized tubes containing SOB medium (Rodina, 1972) and incubated at room temperature (30±2°C) for 4-6 days.

The presence of SOB was indicated by lowering of pH of the medium which was tested by using pH meter. Direct observation of culture under microscope for rod shaped bacteria was also carried out for further confirmation.

Sulphur oxidizing bacterial medium (Rodina, 1972)

Ingredients	
Potassium nitrate	2 g
Ammonium chloride	1 g
Potassium dihydrogen phosphate	2 g
Sodium hydrogen carbonate	2 g
Magnesium sulphate. 7 hydrate	0.8 g
Sodium sulphate. 5 hydrate	5 g
Trace metals	1 ml
Distilled water	1000 ml
pH	6.8-7.0

Sterilization by autoclaving at 121°C for 15 min.

Trace metals	
Ethylene diamine tetraacetic acid, Disodium salt	50 g
Zinc sulphate	2.2 g
Calcium chloride. 2 hydrate	7.34 g
Manganese chloride. 4 hydrate	2.5 g
Cobalt chloride. 6 hydrate	0.5 g
Hexa ammonium molybdate. 4 hydrate	5 g
Copper sulphate. 5 hydrate	0.2 g
Sodium hydroxide	11 g
Distilled water	1000 ml
pH	6

For storage, pH was adjusted to 4, for use pH was readjusted to 6.

3.4.2.4. Sulphate reducing bacteria (SRB)

The MPN technique was used to enumerate SRB. The water and sediment samples were suitably diluted up to 10^{-3} and 10^{-5}, respectively using 1% saline and inoculated into pre-sterilized Sulphate reducing bacterial medium (Rodina, 1972). The inoculated medium was overlayed with a layer of sterile liquid paraffin and incubated at room temperature (30±2°C) for 4-6 days.

The presence of SRB was indicated by the production of H_2S, which gives a black colour to the medium in tubes due to ferrous sulphide formation.

Sulphate reducing bacterial medium (Rodina, 1972)

Ingredients	
Tryptone	10 g
Sodium sulphite	1 g
Ferric ammonium citrate	0.05 g
Distilled water	1000 ml
pH	7.5±0.2

Sterilization by autoclaving at 121°C for 15 min

3.4.2.5. Nitrate reducing bacteria (NRB)

The MPN technique was used to enumerate NRB. The water and sediment samples were suitably diluted up to 10^{-3} and 10^{-5}, respectively using 1% saline, inoculated into sea water nitrate broth (Rodina, 1972) and incubated for 48 h at room temperature (30±2°C). The reduction of NO_3 can terminate in the formation of NO_2 or continue to NH_3 or N_2.

The presence of NRB was indicated by gas production in Durham tubes (if NO_3 is reduced to NH_3 or N_2) or by the appearance of red colour when 1 ml of NO_3 reagent A and 1 ml of NO_3 reagent B was added to the broth culture (if NO_3 is reduced to NO_2).

Sea water nitrate broth (Rodina, 1972)

Ingredients	
Nutrient broth	13 g
Potassium nitrate	1 g
Sea water	500 ml
Distilled water	500 ml
pH	7.6±0.2

Sterilization by autoclaving at 121°C for 15 min.

NO_2 reagent A	
Sulphanilic acid	8 g
Acetic acid (5 N; 1 part of glacial acetic acid to 2.5 parts of water)	1000 ml

NO_3 reagent B	
Dimethyl-α-naphthylamine	5 g
Acetic acid (5 N)	1000 ml

3.4. Isolation, identification and enrichment of bacteria for experimentation

3.5.1. Nitrifying bacteria

3.5.1.1. *Nitrosomonas* sp

The water and sediment samples collected from different shrimp farms were inoculated into modified Winogradsky medium and incubated for few weeks at room temperature (30±2°C). Presence of *Nitrosomonas* sp was tested using Greiss reagent as described in section 3.4.2.2.1.

The isolated samples were further purified by subculturing fortnightly. The isolates of *Nitrosomonas* sp were grown in enrichment media for 4-6 days and used for experimental purposes.

Enrichment medium (Rodina, 1972)

Ingredients	
Ammonium sulphate	1 g
Dipotassium hydrogen phosphate	0.5 g
Sodium chloride	2 g
Magnesium sulphate	0.2 g
Ferrous sulphate. 7 hydrate	0.05 g
Calcium carbonate	6 g
Phenol red	0.01 g
Distilled water	1000 ml
pH	7.6

Sterilization by autoclaving at 121°C for 15 min.

3.5.1.2. *Nitrobacter* sp

The water and sediment samples collected from different shrimp farms were inoculated into Winogradsky medium. Presence of *Nitrobacter* sp was tested

using diphenylamine reagent as described in section 3.4.2.2.2. The isolated samples were further purified by subculturing fortnightly and used for experimental purposes.

3.5.1.3. *Bacillus* sp

The water and sediment samples collected from different shrimp farms were used for the isolation of *Bacillus* sp. Suitably diluted samples were plated on to pre-sterilized TSAS plates and incubated for 24 h at room temperature (30±2°C). A few colonies were picked up and streaked onto TSAS slants for further identification.

TSAS, Trypticase soy agar with 1% sodium chloride (Hi-Media, 1991)

Ingredients	
Pancreatic digest of casein	17 g
Papaic digest of soya meal	3 g
Sodium chloride	10 g
Dibasic potassium phosphate	2.5 g
Agar	15 g
Distilled water	1000 ml
pH	7.3±0.2

Sterilization by autoclaving at 121°C for 15 min.

Bacterial identification was made following the scheme suggested for the gram positive bacteria by Le Chevallier *et al.* (1980). The biochemical tests were performed as per Collins *et al.* (1989).

Aerobic gram positive bacteria with spore forming ability were considered as *Bacillus* sp. They were grown by inoculating into TSBS and incubating overnight at room temperature (30±2°C), and used for experimental purposes.

TSBS, Trypticase soy broth with 1% sodium chloride (Hi-Media, 1991)

Ingredients	
Pancreatic digest of casein	17 g
Papaic digest of soya meal	3 g
Sodium chloride	10 g
Dibasic potassium phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1000 ml
pH	7.3±0.2

Sterilization by autoclaving at 121°C for 15 min.

3.5.2. Isolation of microbial consortium (Microcon) using minimal medium

The water and sediment samples from shrimp farms were inoculated into minimal medium and incubated at room temperature (30±2°C) for 24h. Subculturing of this bacterial mixture was carried out using minimal medium fortnightly

Minimal medium

Ingredients	
Glucose	1 g
Ammonium chloride	0.1 g
Sea water	1000 ml

3.6. Efficacy of commercial bioremediators and products in removing ammonia in microcosm experiments

3.6.1. Efficacy of commercial products including bioremediators

Ten glass aquaria of 40 lit capacity were first rinsed in water, treated with 10 ppm chlorine and then thoroughly washed in clean water to remove traces of

chlorine and dried. Pond sediment collected from shrimp farms was spread uniformly to get a 5 cm thick layer in all the aquaria. Saline water (10ppt) was added into these tanks slowly without disturbing the sediment layer. All the tanks were kept undisturbed for 3 days. Liquid ammonia solution (20%), was then added in to the tanks to get ≥5ppm concentration of ammonia. The tanks were divided into 5 sets of two tanks each. The sets 1, 2, 3 and 4 were seeded respectively with (commercial bioremediators) Biocult, Gasonex, Epicin, and a chemical product Zeolite, at recommended dosage as given below. The levels of ammonia, nitrite and nitrate were daily monitored spectrophotometrically for 8 days following APHA/AWWA/WEF (1998) methods.

Product	Dose prescribed	Dose/tank
Biocult	20kg/ha	0.08g/40 lit
Gasonex	500g/ha	0.002g/40 lit
Epicin	10ppm	0.4g/40 lit
Zeolite	20ppm	0.8g/40 lit

3.6.2. Effect of initial ammonia levels on the efficacy of commercial products

Twenty four glass aquaria of 40 lit capacity were taken and treated as described in 3.6.1. The experimental design and set up for this study, using shrimp pond sediment 5 cm thick and saline water was similar to that of the experiments in 3.6.1. The tanks were divided into 4 sets of six tanks each. In each of the tanks in sets 1-4, the ammonia concentration was adjusted to 1 ppm, 2 ppm, 3 ppm and 4 ppm respectively by the addition of liquid ammonia solution (20%). Each set had six tanks-four as treatment tanks and two as control tanks. The treatment tanks were seeded with commercial products, viz., Biocult and Epicin at recommended dosage in duplicate. The control tanks received no bioproducts. The levels of

ammonia, nitrite and nitrate were daily monitored spectrophotometrically for 8 days following APHA/AWWA/WEF (1998) methods.

3.7. Development of bioremedial measures using indigenous microflora

3.7.1. Efficacy of indigenous flora in removing ammonia in microcosm experiment

Eight glass aquaria of 40 lit capacity in four sets of two tanks each were taken and treated as described in 3.6.1. The experimental design and set up for this study, using shrimp pond sediment 5 cm thick and saline water, was similar to that of the experiments, in 3.6.1. The first 3 sets were seeded respectively with (i) Microcon @ 10^5/ml, (ii) *Nitrosomonas* sp @ 10^5 /ml and *Nitrobacter* sp @ 10^5/ml and (iii) *Bacillus* sp @ 10^5/ml of tank water. The bacterial cells were obtained by growing them in appropriate medium as described earlier in section 3.5, followed by centrifugation at 5000 rpm for 15 min. The washed cells were resuspended separately in physiological saline (0.85% NaCl) and used for seeding into experimental tanks. The fourth set received no inoculum and served as control. The levels of ammonia, nitrite and nitrate were daily monitored spectrophotometrically for 8 days following APHA/AWWA/WEF(1998) methods.

3.7.2. Effect of initial ammonia levels on the efficacy of bioremediatiors with indigenous flora

Forty glass aquaria of 40 lit capacity were taken and treated as described in 3.6.1. The experimental design and set up for this study using shrimp pond sediment 5 cm thick and saline water, was similar to that of the experiments in 3.6.1. The tanks were divided into four sets of ten tanks each with ammonia concentration adjusted to 1ppm, 2 ppm, 3 ppm and 4 ppm, respectively. Each set contained eight treatment tanks (A, B, C and D in duplicate) and two control tanks

(E in duplicate). The treatment tanks were seeded with indigenous microbial cultures as described below :

A - *Bacillus* sp @ 10^5/ml of tank water

B - *Nitrosomonas* sp @ 10^5/ml of tank water and *Nitrobacter* sp @ 10^5/ml of tank water

C - *Nitrosomonas* sp @ 10^5/ml of tank water and *Bacillus* sp @ 10^5/ml of tank water

D - *Nitrosomonas* sp @ 10^5/ml of tank water, *Bacillus* sp @ 10^5/ml of tank water and Microcon @ 10^5/ml of tank water.

The control tanks (E) received no bacterial inoculum.

The preparation of bacterial cells for seeding into experimental tanks was as per 3.7.1. The levels of ammonia, nitrite and nitrate were daily monitored spectrophotometrically for 8 days following APHA/AWWA/WEF (1998) methods.

3.7.3.1. Development of bioremediator (NB Mix) for commercial application

In an attempt to develop a bioremediator with indigenous microflora for commercial application, two bacterial cultures, viz., *Nitrosomonas* sp and *Bacillus* sp were selected. The bacteria were grown in respective medium for 7 and 2 days, respectively as described in 3.5. As a substrate, sand and clay particles from shrimp farm mixed at a ratio of 2:1, sterilized in hot air oven at 100°C for overnight were used. About 20ml each of medium containing *Nitrosomans* sp and *Bacillus* sp were added to 200g of substrate and thoroughly mixed. The substrate containing immobilized bacterial cells, hereafter referred as NB Mix, was then packed in sterile polythene bags and stored at 30±2°C.

Before being used in microcosm experiments, one gram of NB Mix was inoculated into 100 ml sterile diluent to which was added shrimp feed @ 5g/100

ml. This suspension was incubated at 30±2°C for 48 h and seeded into microcosm experimental tanks at 1 ppm level, i.e., 1ml/lit of tank water, to achieve a bacterial density of about 10^5 cells/ml of tank water.

3.7.3.2. Evaluation of the bioremediator, NB Mix

Eight glass aquaria of 40 lit capacity were taken and treated as described in 3.6.1. The experimental design and set up for this study, using shrimp pond sediment and saline water (10ppt), was similar to that of the experiments in 3.6.1. The tanks were stocked with *Oreochromis mossambicus* fingerlings (length 7.4 ± 2.35 cm and weight 6.5 ± 1.8 g) at 4 nos/tank and acclimatized for 5 days during which time they were fed with commercial pelleted feed containing 40% protein twice daily @ 5% of body weight. The initial NH_3, NO_2 and NO_3 concentrations were noted and the tanks were seeded with commercial products, viz., Biocult and Epicin, and the NB Mix as described in 3.6.1. and 3.7.3.1. in duplicate. The control tanks received no inoculum. The fishes were fed twice daily @ 5% of the body weight. The levels of ammonia, nitrite, and nitrate were monitored daily for 8 days following APHA/AWWA/WEF (1998) methods.

3.8. Statistical analyses

One way ANOVA was followed to test the level of significance among treatments and critical difference calculated to examine which of the treatments varied significantly. Students 't' – test was followed to test the significance of difference between the bacteriological as well as physico-chemical parameters of water and soil according to the type of system and days of culture (Snedecor and Cochran, 1962).

Chapter - 4

RESULTS

4. RESULTS

4.1. Quantification of bacteria of different physiological characteristics in shrimp culture systems

The results of the bacterial counts are presented in Tables 2 and 3. The results of statistical interpretation are presented in Table 5.

4.1.1. Total heterotrophic bacterial counts (THCs)

The THCs of pond water were highest in semi-intensive system with a peak of 1.02 x 10^7 cfu/ml at DOC>120. The lowest count of 1.00 x 10^2 cfu/ml was recorded in traditional system at DOC 90-120 (Tables 2 and 3). There existed significant differences ($P<0.05$) in THCs of pond water between traditional and other systems. The differences in other systems were insignificant (Tables 2, 3 and 5).

The THCs of sediment were highest in stagnant pond culture system with a peak of 1.7 x 10^7 cfu/g at DOC<30. The traditional system recorded the lowest of 1.00 x 10^5 cfu/g at DOC 90-120 (Tables 2 and 3). The sediment THCs between semi-intensive and traditional systems, stagnant pond culture and traditional systems, and stagnant pond culture and modified extensive systems differed significantly ($P<0.05$); while others differed insignificantly (Tables 2, 3 and 5).

4.1.2. Nitrifying bacteria

4.1.2.1. *Nitrosomonas* sp

The counts of *Nitrosomonas* sp were higher in pond sediment than in pond water of all culture systems. The highest count of *Nitrosomonas* sp in water was recorded in traditional system with a peak of MPN 9.2 x 10^4/100 ml at DOC>120. The sediment of modified extensive culture system recorded the highest of MPN 9.2 x 10^4 /g at DOC<30 (Tables 2 and 3).

Significant differences ($P<0.05$) in *Nitrosomonas* sp counts of pond water between semi-intensive and traditional systems, stagnant pond culture and traditional systems, and stagnant pond culture and modified extensive systems were noticed. The *Nitrosomonas* sp counts in pond sediment samples of stagnant pond culture system varied significantly ($P<0.05$) with the counts of semi-intensive and traditional systems (Tables 2, 3 and 5).

4.1.2.2. *Nitrobacter* sp

The counts of *Nitrobacter* sp in pond water were higher when compared to *Nitrosomonas* sp in all the culture systems. The highest count recorded was MPN 9×10^4/100 ml. The counts of *Nitrobacter* sp recorded highest in pond sediment samples of stagnant pond culture system with a peak of MPN 5.42×10^4/g at DOC 30-60 (Tables 2 and 3).

The counts of *Nitrobacter* sp in pond water samples of semi-intensive system varied significantly ($P<0.05$) with the counts of improved traditional systems, while others differed insignificantly ($P>0.05$). There existed significant differences ($P<0.05$) in *Nitrobacter* sp counts in pond sediment samples between semi-intensive and improved traditional systems, semi-intensive and stagnant pond culture systems, semi-intensive and modified extensive systems, improved traditional and traditional systems, and stagnant pond culture and traditional systems (Tables 2, 3 and 5).

4.1.3. Sulphur oxidizing bacteria (SOB)

The counts of SOB in pond water were higher in improved traditional system with a peak of MPN 3.5×10^4/ 100 ml at DOC<30. In sediment, the highest was in semi-intensive system with a peak of MPN 9.2×10^3/g at DOC < 30 and 60-90, respectively (Tables 2 and 3).

The SOB counts in pond water of improved traditional system varied significantly ($P<0.05$) with those of traditional system. The counts of SOB, on the

other hand, differed insignificantly (P>0.05) with respect to other systems (Tables 2, 3 and 5).

4.1.4. Sulphate reducing bacteria (SRB)

The counts of sulphate reducers were always high in pond sediment than in pond water samples. The highest count in pond water was recorded in traditional system with a peak of MPN $2.5x10^5$/100ml at DOC 30-60. The sediment samples of stagnant pond culture system recorded the highest SRB, a peak of MPN $3.48x10^4$/g at DOC 30-60 (Tables 2 and 3).

The SRB counts in pond water of traditional system varied significantly (P<0.05) with the counts of semi-intensive, stagnant pond culture and improved traditional systems. There existed significant differences (P<0.05) in SRB counts of pond sediment samples between semi-intensive and improved traditional systems, semi-intensive and stagnant pond culture systems, semi-intensive and traditional systems, and traditional and modified extensive systems (Tables 2, 3 and 5).

4.1.5. Nitrate reducing bacteria (NRB)

The counts of NRB in pond water were highest in traditional system with a peak of MPN $7x10^5$/100 ml at DOC<30. In pond sediment, the stagnant pond culture system recorded a peak MPN $9.2x10^4$/g at DOC 30-60 (Tables 2 and 3).

The pond water NRB counts of modified extensive system varied significantly (P<0.05) with the counts of semi-intensive and stagnant pond culture systems. Significant differences (P<0.05) in NRB counts of pond sediment samples between semi-intensive and stagnant pond culture systems, semi-intensive and modified extensive systems, improved traditional and stagnant pond culture systems, improved traditional and traditional systems, improved traditional and modified extensive systems, stagnant pond culture and traditional systems, and modified extensive and traditional systems were noted. Also, the NRB counts of

first 60 DOC in sediment samples differed significantly ($P<0.05$) with those of the later, i.e., >60 DOC (Table 2, 3 and 5).

4.2. Physico-chemical characteristics of shrimp culture systems

4.2.1. Temperature

The water temperature of different shrimp farming systems varied between 21 and 32°C throughout the culture period (Table 4).

4.2.2. pH

The pH of water varied among different culture systems. The lowest value of 6.8 was recorded in stagnant pond culture system. The highest value of 8.8 was recorded in semi-intensive system (Table 4). The pH of sediment samples also varied considerably among different culture systems. The lowest value recorded was 7.9 in the improved traditional and modified extensive systems, while the highest value was 9.6 in the traditional system (Table 4).

4.2.3. Salinity

The salinity of pond water fluctuated between 1 and 21 ppt among different shrimp farming systems with an average of about above 5 ppt in traditional system and about $\geq$ 10 ppt in other systems. The lowest value of 1 ppt was recorded in the traditional system at DOC<30. The highest value of 21 ppt was recorded in the stagnant pond culture system at DOC 30-60 (Table 4).

4.2.4. Ammonia-nitrogen

The ammonia-nitrogen levels were highest in the semi-intensive system with a peak of 600 μg NH_3-N/l towards the end of culture and the lowest in traditional system, 36.22 μg NH_3-N/l at DOC < 30. The mean values of NH_3-N were found to increase with DOC (Table 4). Significant differences in NH_3 levels were noticed among the culture systems ($P<0.05$). The levels of NH_3-N between semi-intensive and traditional, stagnant pond culture and improved traditional,

improved traditional and traditional, stagnant pond culture and traditional and improved traditional and modified extensive systems were significantly ($P<0.05$) differed (Tables 4 and 5).

4.2.5. Nitrite-nitrogen

The nitrite-nitrogen levels were highest in traditional system with a peak of 4.28 µg at. NO_2-N/l at DOC 60-90. The lowest level of 0.0185 µg at. NO_2-N/l was recorded in the traditional system at DOC < 30 (Table 4). There existed a significant ($P<0.05$) difference in NO_2-N levels between semi-intensive and stagnant pond culture systems. The differences in their levels in other systems were insignificant ($P>0.05$). Also, the NO_2-N levels of first 60 DOC differed significantly ($P<0.05$) than those of later, i.e., 60-120 DOC (Tables 4 and 5).

4.2.6. Nitrate-nitrogen

The nitrate-nitrogen levels were highest in traditional system with a peak of 2.372 µg at. NO_3-N/l at DOC<30 and lowest, 0.2 µg at. NO_3-N/l in stagnant pond culture system at DOC 30-60 (Table 4). Significant differences ($P<0.05$) in NO_3-N levels between traditional and stagnant pond culture systems, traditional and modified extensive systems, traditional and semi-intensive systems, and stagnant pond culture and semi-intensive systems were observed (Tables 4 and 5).

4.3. Efficacy of commercial bioremediators and products in removing total ammoniacal nitrogen (TAN) in microcosm experiment

4.3.1. Efficacy of commercial products including bioremediators

Among the different commercial bioremediators and products tested in reducing TAN from 5 ppm, Biocult was the most efficient, which removed 79.3% of NH_3 in 7 days. This was followed by Gasonex, Epicin and Zeolite with a removal of 69.68, 65 and 63.18% NH_3, respectively. The TAN removal was only

50.6% in control experiment (Fig 2a; Table 6). The differences in the levels of TAN in all tanks, were however, statistically insignificant ($P>0.05$) (Table 8).

The nitrite levels increased in all the experimental and control tanks with the highest increase of 23.2% in tank treated with Zeolite and the lowest increase of 4.3% in tank treated with Epicin (Fig 2b and Table 6). The nitrate levels also increased in all the experimental and control tanks with the highest increase of 82.67% in tanks treated with Gasonex and the lowest increase of 57.4% in tanks treated with Biocult (Fig 2c and Table 6). The nitrite and nitrate levels in all the experimental and control tanks, however, differed ($P>0.05$) insignificantly (Table 8).

4.3.2. Effect of initial ammonia levels on the efficacy of commercial products including bioremediators

Among the commercial products tried (Biocult and Epicin), Biocult was efficient in removing 78-90% TAN at all NH_3 levels. Epicin was also equally efficient, removing 81-86% TAN at 1-3 ppm initial levels of NH_3. However, at 4 ppm NH_3 levels, it could remove only 60% of TAN (Figs. 3a, 4a, 5a and 6a; Table 6). There was a general reduction in TAN removal rate with increase in initial NH_3 levels in the control tanks. At 1 ppm. NH_3 levels, nearly 50% of TAN was removed, while in others it was in the range of 28-37% (Figs. 3a, 4a, 5a and 6a; Table 6). The TAN removal rates of Biocult and Epicin treated tanks and control tanks differed significantly ($P<0.05$) at all the four different initial NH_3 levels (Table 8).

The nitrite levels increased in all the experimental tanks treated with commercial products and also in control tanks. The increase in percentage of NO_2 in Biocult treated tanks was highest at 4 ppm level of NH_3 and least at 1 ppm level of NH_3. The increase in percentage of NO_2 in Epicin treated tanks was highest at 3 ppm level of NH_3 and least at 2 ppm level of NH_3. The increase in percentage of

NO_2 in control tanks was highest at 1 ppm level of NH_3 and least at 4 ppm level of NH_3 (Figs. 3b, 4b, 5b, 6b ; Table 6).

Significant differences were noticed among treatments and also among various NH_3 levels ($P < 0.05$). The levels of NO_2 in Epicin treated tanks and control tanks with 1 ppm level of NH_3 were significantly different ($P<0.05$) from those of 2, 3 and 4ppm level of NH_3. Biocult treated tanks with 1 ppm NH_3 had significantly ($P<0.05$) high NO_2 than Epicin treated and control tanks. Statistically significant differences ($P<0.05$) existed between the levels of NO_2 at 2, 3 and 4 ppm level of NH_3 between control tanks and tanks treated with Biocult and Epicin (Table 8).

The nitrate levels increased in all the treatment tanks, but showed a general decreasing trend in control tanks. The increase in percentage of NO_3 in Biocult treated tanks was highest at 2 ppm level of NH_3 and least at 4 ppm level of NH_3. The increase in percentage of NO_3 in Epicin treated tanks was highest at 1 ppm level of NH_3 and least at 2 ppm level of NH_3 (Figs. 3c, 4c, 5c, 6c; Table 6). Among the tanks with varied NH_3 concentrations, the levels of NO_3 between the control and treatment tanks (Table 8) differed insignificantly ($P>0.05$).

4.4. Development of bioremedial measures using indigenous microflora

4.4.1. Efficacy of indigenous flora in removing ammonia in microcosm experiment

Among the different indigenous bacterial flora tried in reducing TAN from 5 ppm level, the mixture of *Nitrosomonas* sp and *Nitrobacter* sp was the most efficient and capable of removing about 96.5% of TAN, followed by *Bacillus* sp and Microcon with a removal of 95.73% and 70.84% TAN, respectively, while in control tanks only 50% of TAN was removed (Fig 7a, Table 6). There were no

significant differences ($P>0.05$) in TAN removal rates between the seeded tanks and control tanks at 5 ppm level of NH_3 (Table 8).

A slight decrease in nitrite levels of about 7% was seen in *Bacillus* sp seeded tanks although with fluctuations, while it increased in all the other experimental and control tanks. The highest increase in NO_2 of 28.21% was recorded in tanks seeded with Microcon and control being the least with only 17.2% increase in NO_2 (Fig 7b, Table 6). There was a increase in NO_3 levels in all the experimental and control tanks. The increase was highest (84.3%) in Microcon seeded tanks. The increase was only 1.5% in *Bacillus* sp seeded tanks (Fig 7c, Table 6). The levels of NO_2 and NO_3 at 5 ppm level of NH_3 were statistically insignificant ($P>0.05$) in all the experimental and control tanks (Table 8).

4.4.2. Effect of initial ammonia levels on the efficacy of bioremediators with indigenous flora

The results of the addition of indigenous microflora on the removal of TAN in microcosm experiments are presented in Figs. 8a, 9a, 10a and 11a, and Table 6. The indigenous microflora in combination or alone were capable of removal of TAN to the tune of > 85-99% within a week. *Bacillus* sp was capable of removal of 88-95% TAN within a week. Significant differences ($P<0.05$) in TAN removal were noticed with variation in initial NH_3 levels. The reduction of TAN in tanks with initial NH_3 of 1 and 2 ppm varied markedly ($P<0.05$) with those of 3 and 4 ppm level of NH_3 (Figs. 8a, 9a, 10a and 11a; Tables 6 and 8).

The mixture of *Nitrobacter* sp and *Nitrosomonas* sp worked well ($P<0.05$) when the initial NH_3 levels were low, i.e., 1 ppm and 2 ppm, removing more than 90-95% of TAN. At 3 and 4 ppm levels, the removal of TAN was 86.5% and 85.8%, respectively. The initial ammonia level did have a significant effect ($P<0.05$) on the removal of TAN (Figs. 8a, 9a, 10a, and 11a; Tables 6 and 8).

The combination of *Nitrosomonas* sp, *Bacillus* sp and microbial consortium (Microcon) was the most effective among all, removing more than 97.5% TAN.

The initial ammonia levels did not have any effect ($P>0.05$) on the TAN removal rate (Figs. 8a, 9a, 10a and 11a; Tables 6 and 8).

The combination of *Nitrosomonas* sp and *Bacillus* sp was also equally effective, removing about above 96% of TAN. There existed significant differences ($P<0.05$) in the reduction of TAN at different initial NH_3 levels (Figs. 8a, 9a, 10a and 11a; Tables 6 and 8). There was a general decrease in TAN removal with increase in initial NH_3 level. The initial NH_3 levels had a significant effect ($P<0.05$) on TAN removal (Tables 6 and 8). The TAN removal rate at 2 ppm initial level of NH_3 in control tanks was significantly different ($P<0.05$) from those of other levels (Tables 6 and 8).

The treatments with a combination of *Nitrosomonas* sp, *Bacillus* sp and Microcon showed significantly differences ($P<0.05$) with those of treatments with *Bacillus* sp alone, *Nitrobacter* sp and *Nitrosomonas* sp mixture and control at 3 ppm NH_3 level. Likewise, the mixture of *Nitrosomonas* sp and *Bacillus* sp differed significantly ($P<0.05$) with control (Tables 6 and 8).

The nitrite levels increased in all the experimental tanks, except the tanks seeded with a combination of *Nitrosomonas* sp, *Bacillus* sp and Microcon at 1 ppm and 3 ppm levels of NH_3 (Figs. 8b, 9b, 10b and 11b; Table 6). The increase in NO_2 in *Bacillus* sp seeded tanks was the highest (99%) at 1 ppm level of NH_3 and the least (91.3%) at 3 ppm level of NH_3. The increase in NO_2 in tanks seeded with *Nitrosomonas* sp and *Nitrobacter* sp mixture was high (98.5%) at 1 ppm level of NH_3 and the least at 3 ppm level of NH_3. There existed significant differences ($P<0.05$) in the levels of NO_2 in *Bacillus* sp seeded tanks and in tanks seeded with *Nitrosomonas* and *Nitrobacter* sp mixture between 1 and 2 ppm level of NH_3 and those containing 3 ppm and 4 ppm level of NH_3 (Figs. 8b, 9b, 10b and 11b; Tables 6 and 8). The increase in NO_2 was high (94.7%) in tanks seeded with a mixture of *Nitrosomonas* sp, *Bacillus* sp and Microcon at 4 ppm level of NH_3 and the least (-86.9%) at 1 ppm level of NH_3. The level of NO_2 in tanks seeded with a mixture of *Nitrosomonas* sp, *Bacillus* sp and Microcon at 1 ppm level of NH_3 was

significantly different ($P<0.05$) from those of tanks containing 4 ppm of NH_3 (Figs. 8b, 9b, 10b, 11b; Tables 6 and 8). The increase in NO_2 was high (99.9%) in tanks seeded with a mixture of *Bacillus* sp and *Nitrosomonas* sp at 1 and 4 ppm level of NH_3 and the least (92%) at 2 ppm level of NH_3. The level of NO_2 in tanks seeded with a mixture of *Nitrosomonas* sp and *Bacillus* sp at 3 ppm level of NH_3 was significantly different ($P<0.05$) from those of tanks with 1, 2 and 4 ppm levels of NH_3 (Figs. 8b, 9b, 10b, 11b; Tables 6 and 8). The levels of NO_2 in control tanks with 1 ppm NH_3 was significantly different ($P<0.05$) from those of tanks containing 2, 3 and 4 ppm of NH_3 (Tables 6 and 8).

At 1 ppm NH_3 level, the levels of NO_2 in tanks seeded with a mixture of *Bacillus* sp, *Nitrosomonas* sp and Microcon and a mixture of *Bacillus* sp and *Nitrosomonas* sp were significantly different ($P<0.05$) from other tanks (Tables 6 and 8).

At 2 ppm level of NH_3, the NO_2 level in tanks seeded with a mixture of *Bacillus* sp, *Nitrosomonas* sp and Microcon was significantly different ($P<0.05$) from other treatments excluding control tanks (Tables 6 and 8).

At 3 and 4 ppm levels of NH_3, the NO_2 levels in tanks seeded with a mixture of *Bacillus* sp, *Nitrosomonas* sp and Microcon and in control tanks varied significantly ($P<0.05$) from those of other tanks (Tables 6 and 8).

The nitrate levels varied in all the treatment tanks seeded with indigenous flora and decreased in the control tanks (Figs. 8c, 9c, 10c, 11c; Table 6). The tanks seeded with *Bacillus* sp, *Nitrosomonas* sp and *Nitrobacter* sp mixture, *Bacillus* sp and *Nitrosomonas* sp and Microcon mixture, *Bacillus* sp and *Nitrosomonas* sp mixture and the control tanks at respective NH_3 concentrations of 1 ppm, 2 ppm, 2 ppm, 1 ppm and 1 ppm had highest levels of NO_3 (Figs. 8c, 9c, 10c, 11c; Table 6). Significant differences ($P<0.05$) existed among treatments and also among NH_3 concentrations. The NO_3 levels in the control tanks were significantly different ($P<0.05$) from those of seeded tanks at all the four different initial NH_3 levels. At 1 ppm and 2 ppm levels of NH_3, the observed levels of NO_3 in tanks seeded with a

mixture of *Bacillus* sp and *Nitrosomonas* sp was significantly different ($P<0.05$) from those of tanks seeded with a mixture of *Nitrosomonas* sp and *Nitrobacter* sp and those seeded with *Bacillus* sp (Tables 6 and 8).

4.4.3. Evaluation of the bioremediator (NB Mix) in simulated pond condition

The efficacy of *Nitrosomonas* sp and *Bacillus* sp mixture immobilized in sand and clay (NB Mix), compared to Biocult, Epicin and control was carried out in simulated pond conditions in the laboratory. The TAN levels increased in all the tanks despite the use of bioremediators (Fig 12a; Table 7). The increase was more in Biocult (45.85%) and least in Epicin (42.32%) and NB Mix (42.29%) treated tanks (Fig 12a; Table 7). There existed no significant difference ($P>0.05$) in the levels of TAN in all the treated and control tanks (Table 8).

The NO_2 and NO_3 levels increased in all the treated tanks along with control. The increase in NO_2 was maximum in Epicin (58.12%) and least in Biocult (26.25%) treated tanks, while the increase in NO_3 was maximum in Biocult (79.65%) and least in NB Mix (49.42%) treated tanks (Figs. 12b and 12c; Table 7). Significant differences ($P<0.05$) existed in NO_2 levels between the control and treated tanks. But, the levels of NO_3 showed no significant differences ($P>0.05$) between the control and treated tanks (Table 8).

Table 2. Bacteriological characteristics of different shrimp culture systems.

System	Heterotrophic count/ml/g		*Nitrosomonas* count/100ml/g		*Nitrobacter* count/100ml/g		Sulphate Reducing Bacteria count/100ml/g		Nitrate Reducing Bacteria count/100ml/g		Sulphur Oxidizing Bacteria count/g/100ml	
Water	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Semi intensive	$2.1x10^5$ – $1.02x10^7$	2.37±2.75 $x10^6$	0 – $2.5x10^3$	6.7± $8.31x10^2$	2x10 – $9x10^4$	1.78± $2.86x10^4$	5x10 – $3.5x10^4$	8.08± $10.66x10^3$	0 – $3.5x10^4$	7.83± $12.3x10^3$	0 – $3.5x10^4$	3.51± $10.49x10^3$
Modified extensive	$3x10^6$- $1.21x10^6$	1.94±0.74 x 10^6	0 – $1.3x10^3$	4.06± $4.62x10^2$	$11x10^1$ – $8x10^4$	4.21± $3.45x10^4$	$1.41x10^3$ – $2.4x10^4$	1.16± $1.01x10^4$	$1.1x10^4$ – $4.6x10^4$	2.96± $1.4x10^4$	0	0
Stagnant pond culture	$3.3x10^5$ – $3x10^6$	1.25±1.03 $x10^6$	0 – $3.5x10^2$	1.34± $1.15x10^2$	5x10 – $9x10^4$	2.48± $3.04x10^4$	0 – $2.1x10^4$	6.09 $\pm 7.07x10^3$	$1.2x10^2$ – $3.5x10^4$	1.10± $1.05x10^4$	0	0
Improved traditional	$8x10^5$ – $2.5x10^6$	1.75±0.63 $x10^6$	0 – $1.1x10^3$	3.8± $5.09x10^2$	$1.6x10^4$ – $9x10^4$	4.73± $3.12x10^4$	$1.7x10^2$ – $7x10^2$	4.35± $2.65x10^2$	$2.8x10^2$ – $2.4x10^4$	1.21± $1.18x10^4$	$2.1x10^4$ $-3.5x10^4$	2.56± $6.59x10^4$
Traditional	$1x10^2$ – $1.41x10^6$	3.83±5.2 $x10^5$	0 – $9.2x10^4$	1.00± $2.40x10^4$	0 – $8x10^4$	1.98± $2.75x10^4$	$5x10^2$ – $2.5x10^5$	3.88± $6.51x10^4$	$1.5x10^2$ – $7x10^5$	6.72± $16.7x10^4$	0 – $3.5x10^2$	6.65± $10.91x10^1$
Soil												
Semi intensive	$5.8x10^5$ – $5.3x10^6$	2.68±1.64 $x10^6$	$2.3x10^1$ – $9.2x10^3$	4.04± $4.75x10^3$	2 – $9.2x10^3$	1.39± $2.76x10^3$	$1.75x10^2$ – $2.4x10^4$	6.67± $7.93x10^3$	$4.3x10^1$ – $9.2x10^3$	2.70± $2.75x10^3$	0 – $9.2x10^3$	7.07± $24.57x10^2$
Modified extensive	$1.38x10^7$ $-3.9x10^6$	7.45±0.34 $x10^6$	$2.3x10^2$ – $9.2x10^4$	1.9±3.6x 10^4	$7x10^1$ – $2.4x10^4$	1.38± $0.97x10^4$	$1.1x10^2$ – $1.6x10^4$	1.19±0.93 $x10^4$	$2.4x10^3$ – $3.48x10^4$	1.53± $1.22x10^4$	0	0
Stagnant pond culture	$7.25x10^5$ – $1.7x10^7$	8.23±4.86 $x10^6$	$5.42x10^3$ – $1.6x10^4$	8.75± $3.91x10^3$	$1.4x10^2$ – $5.42x10^4$	1.68± $2.06x10^4$	$2.4x10^3$ – $3.48x10^4$	1.10± $1.08x10^4$	$4.6x10^2$ – $9.2x10^4$	3.48± $3.29x10^4$	0	0
Improved traditional	$1.02x10^6$ – $7.1x10^6$	3.88±2.21 $x10^6$	$9.2x10^2$ – $1.6x10^4$	5.23± $6.24x10^3$	$1.6x10^3$ – $5.42x10^3$	3.02± $1.58x10^3$	$5.42x10^3$ – $1.6x10^4$	1.02± $0.43x10^4$	$2.8x10^1$ – $3.48x10^3$	1.19± $1.61x10^3$	0 – $9.2x10^2$	5.01± $3.27x10^2$
Traditional	$1x10^5$ – $1.05x10^7$	4.71±3.77 $x10^6$	2.3 – $1.6x10^4$	2.53± $4.48x10^3$	0 – $9.2x10^3$	1.41± $2.49x10^3$	$2.4x10^3$ – $2.4x10^4$	1.39± $7.26x10^4$	0 – $9.2x10^3$	3.22± $2.42x10^3$	0	0

Table 3. Bacteriology of shrimp culture systems as affected by days of culture (DOC).

DOC	Heterotrophic count/ml/g		*Nitrosomonas* count/100ml/g		*Nitrobacter* count/100ml/g		Sulphate Reducing Bacteria count/100ml/g		Nitrate Reducing Bacteria count/100ml/g		Sulphur Oxidizing Bacteria count/gm/100ml	
Water	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
< 30	$9x10^4$– $2.5x10^6$	8.54± $8.84x10^5$	0-$1.7x10^3$	2.65± $5.23x10^2$	50-$9x10^4$	2.00± $2.75x10^4$	$1.1x10^2$ – $8x10^3$	2.79± $3.13x10^3$	0-$7x10^5$	8.77± $20.99x10^4$	0 - $3.5x10^4$	6.44± $1.15x10^3$
30-60	$3.3x10^5$ –$3x10^6$	1.33± $1.01x10^6$	80- $2.5x10^3$	7.74± $9.38x10^2$	0-$9x10^4$	1.40± $2.73x10^4$	0-$2.5x10^5$	5.56± $7.71x10^4$	$1.2x10^2$ – $3.5x10^4$	1.03± $1.18x10^4$	0- $3.5x10^2$	6±12.2x10
60-90	$3.22x10^5$ –$9.8x10^5$	6.77± $9.68x10^5$	0-$2.5x10^3$	4.90± $9.02x10^2$	$1.4x10^3$ – $8x10^4$	3.49± $2.69x10^4$	$1.3x10^3$ – $5.5x10^4$	1.43± $1.94x10^4$	0-2.4 $x10^4$	6.67± $8.43x10^3$	0 – $3.5x10^4$	5.88 ±$13.04x10^3$
90-120	$1x10^2$– $4.5x10^6$	1.52± $1.15x10^6$	0-$1.1x10^4$	2.785 ±$3.46x10^3$	0-$35x10^4$	5.42± $12.09x10^3$	$2.5x10^3$ – $4.5x10^4$	1.28± $1.43x10^4$	$2x10^2$ $3.5x10^4$	1.19± $1.40x10^4$	0	0
>120	$1x10^6$– $1.02x10^7$	3.09± $2.75x10^6$	20- $9.2x10^4$	3.67± $3.87x10^4$	$3.5x10^2$ – $9x10^4$	5.13± $3.59x10^4$	5x10 – $3.5x10^4$	1.23± $1.34x10^4$	7x10- $3.5x10^4$	1.71± $1.49x10^4$	0	0
Soil												
< 30	$2.1x10^5$– $1.7x10^7$	5.82± $4.47x10^6$	$2.3x10^1$ – $9.2x10^4$	1.27± $2.58x10^4$	2 – $3.48x10^3$	1.01± $1.29x10^3$	$3.48x10^2$ – $2.4x10^4$	7.96± $7.07x10^3$	$2.8x10^1$ – $3.48x10^4$	9.08± $1.30x10^3$	0- $9.2x10^3$	1.82± $3.11x10^3$
30-60	$7.25x10^5$– $1x10^7$	5.47 ±$3.83x10^6$	$2.3x10^2$– $1.6x10^4$	4.18± $4.69x10^3$	0- $5.42x10^4$	1.03± $1.86x10^4$	$2.4x10^3$ – $3.48x10^4$	1.23± $1.68x10^4$	$7.5x10^1$ – $9.2x10^4$	1.65± $2.98x10^4$	0	0
60-90	$1.5x10^5$– $7.35x10^6$	3.15 ±$3.06x10^6$	$2.3x10^1$ – $9.2x10^3$	3.95± $3.87x10^3$	$2.6x10^1$ – $9.2x10^3$	4.22± $4.14x10^3$	$1.75x10^2$ – $2.4x10^4$	8.26± $8.78x10^3$	$4.3x10^1$– $5.42x10^3$	3.18± $1.87x10^3$	0 – $9.2x10^3$	1.84x± $3.68x10^3$
90-120	$1x10^5$– $4.3x10^6$	1.90 ±$1.77x10^6$	$1.41x10^2$ –$1.6x10^4$	4.62± $5.97x10^3$	0 – 5.42 x 10^3	9.71± $1.99x10^2$	$3.45x10^3$ – $2.4x10^4$	1.00± $7.65x10^4$	$2.8x10^1$ – $9.2x10^3$	4.47± $2.96x10^3$	0	
>120	$1.6x10^6$– $1.05x10^7$	4.8 ±$3.11x10^6$	$5.42x10^2$ – $1.6x10^4$	4.82± $5.87x10^3$	$0.4x10^2$ – $9.2x10^3$	2.58± $3.33x10^3$	$2.4x10^3$ – $2.4x10^4$	1.14± $7.76x10^4$	$2.4x10^2$ – $5.42x10^3$	1.64± $1.94x10^3$	0	

Table 4. Physico-chemical characteristics of shrimp culture systems.

	NH_3 (µg NH_3-N/l)		NO_2 (µg at. NO_2 -N/l)		NO_3 (µg at. NO_3-N/l)		Salinity (ppt)		pH		Temperature (°C)	
DOC	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
< 30	36.22 – 62.19	45.98 ± 9.21	0.0185 – 2.906	0.698± 0.917	0.214 – 2.372	1.054± 0.693	1 – 15	7.64 ± 4.5	Water: 7.7 – 8 .8 Soil: 7.9 – 9.6	8.2±0.3 8.75±0.55	21 – 32	26.6 ± 4.31
30-60	65.31 – 222.04	119.89 ± 51.36	0.112 – 1.525	0.958± 0.448	0.2 – 0.688	0.351 ± 0.147	2 – 21	13.18± 6.57	Water: 6.8 – 8.6 Soil: 8.0 – 9.6	7.8±0.64 9±0.52	26 – 32	29.2± 2.12
60-90	100.16 – 252.03	164.94 ± 62.1	0.8085 – 4.28	1.969 ± 1.39	0.42 – 1.41	0.728 ± 0.396	10 - 17	12.75 ± 2.58	Water: 7.3 – 8 .8 Soil: 8.2 – 8.9	8.12±0.57 8.65 ±0.27	28 – 31	29.5 ±1.5
90-120	45.74 – 282.0	115.56 ± 97.9	1.0135 – 3.119	1.726 ± 0.755	0.375 – 1.587	0.783 ± 0.435	2 – 14.5	7.35 ± 3.82	Water: 7.3 – 8.1 Soil: 8.2 – 9.4	7.8±0.25 8.95±0.45	26 – 30	28± 1.52
>120	52.85 – 600.0	240.05 ± 254.5	1.509 – 1.74	1.66 ± 0.10	0.62 – 0.718	0.675 ± 0.04	2 – 14.5	7.5 ± 4.93	Water: 7.6– 8.6 Soil: 8.8 – 9.1	8.1±0.33 9±0.11	25 – 30.5	27.6 ±2.63
System												
Semi intensive	50-600.0	215.5± 221.6	0.954 – 2.30	1.515± 0.496	0.375– 0.613	0.492± 0.103	6 - 15	11.3± 2.5	Water: 7.6 – 8.8 Soil: 8.2 – 9.4	8.25±0.38 8.8±0.32	28 – 30.5	29.4± 1.01
Modified extensive	36.38- 152.18	90.2± 40	0.324 – 1.825	1.168± 0.5	0.24 - 0.727	0.458± 0.17	10-12.5	10.83± 1.17	Water: 7.6 – 7.7 Soil: 7.9 – 8.1	7.63±0.047 8±1	30 - 31	30.33 ±0.47
Stagnant pond culture	56.38 – 123.25	93.275± 24.03	0.4 – 1.38	0.764± 0.33	0.2 – 0.56	0.347± 0.13	7 – 21	14.14± 4.48	Water: 6.8 – 8.6 Soil: 8.0 – 9.4	7.7±0.63 8.5±0.44	29 – 32	30.3± 1.03
Improved traditional	222.04	222.04	0.948	0.948	0.272	0.272	8 – 11	9.0± 1	Water: 7.9 – 8.3 Soil: 7.9 – 9.0	8.07±0.14 8.25±0.43	28.5 – 32	30.4± 1.29
Traditional	36.22 – 252.03	86.84± 60.66	0.0185 – 4.28	1.377± 1.14	0.207 – 2.372	0.954± 0.565	1 – 20	5.466± 6.1	Water: 7.2 – 8.7 Soil: 8.8 – 9.6	7.9±0.43 9.3±0.26	21 – 27	24.4± 2.32

Table 5. t-values computed for different bacteriological and physico-chemical parameters of shrimp farming systems.

Parameter	Water			Soil		
	d.f.	t_{cal}	t_{crit}	d.f.	t_{cal}	t_{crit}
1) THC						
SI vs IT	13	0.31	1.77	13	1.07	1.77
SI vs SPC	13	0.57	1.77	13	1.42	1.77
SI vs T	18	3.90[a]	1.73	18	0.43	1.73
SI vs ME	13	0.50	1.77	14	2.95[a]	1.76
IT vs SPC	6	1.04	1.94	8	1.04	1.86
IT vs T	11	1.86[a]	1.80	11	0.41	1.80
IT vs ME	6	0.20	1.94	7	2.05[a]	1.90
SPC vs T	11	2.80[a]	1.80	13	1.31	1.77
SPC vs ME	6	1.32	1.94	9	0.43	1.83
T vs ME	11	2.10[a]	1.80	12	1.55	1.78
<60 DOC vs >60 DOC	30	0.07	1.70	13	1.50	1.70
2) *Nitrosomonas* sp						
SI vs IT	9	0.21	1.83	15	0. 98	1.75
SI vs SPC	13	1.00	1.77	17	2.62[a]	1.74
SI vs T	19	2.27[a]	1.73	24	0.23	1.71
SI vs ME	11	0.20	1.80	16	0.32	1.75
IT vs SPC	6	0.57	1.94	8	1.84	1.86
IT vs T	10	1.62	1.81	15	1.25	1.75
IT vs ME	4	0.76	2.13	7	0.46	1.90
SPC vs T	16	4.00[a]	1.75	17	3.10[a]	1.74
SPC vs ME	8	1.92[a]	1.86	9	1.64	1.83
T vs ME	14	1.74	1.76	16	0.50	1.75
<60 DOC vs >60 DOC	31	1.65	1.7	38	0.04	1.70

Continued...

Table 5 continuation

Parameter	Water			Soil		
	d.f.	t_{cal}	t_{crit}	d.f.	t_{cal}	t_{crit}
3) *Nitrobacter* sp						
SI vs IT	11	1.81[a]	1.8	14	2.48[a]	1.76
SI vs SPC	15	0.09	1.75	15	3.02[a]	1.75
SI vs T	21	0.15	1.72	22	0.45	1.72
SI vs ME	13	1.11	1.77	15	2.88[a]	1.75
IT vs SPC	8	1.31	1.86	7	0.61	1.90
IT vs T	14	1.35	1.76	14	1.87[a]	1.76
IT vs ME	6	0.69	1.94	7	0.52	1.90
SPC vs T	18	0.37	1.73	15	2.42[a]	1.75
SPC vs ME	10	0.65	1.81	8	0.06	1.86
T vs ME	16	0.86	1.75	15	1.15	1.75
<60 DOC vs >60 DOC	36	0.55	1.70	35	0.27	1.70
4) Sulphur oxidizing bacteria						
SI vs IT	3	0.29	2.35	3	1.7	2.35
SI vs T	5	1.35	2.02			
T vs IT	6	7.20[a]	1.94			
<60 DOC vs >60 DOC	8	0.13	1.86	3	0.23	2.35
5) Nitrate reducing bacteria						
SI vs IT	8	0.18	1.86	13	0.96	1.77
SI vs SPC	13	0.96	1.77	16	2.57[a]	1.75
SI vs T	22	1.61	1.72	22	1.50	1.72
SI vs ME	11	2.40[a]	1.80	15	2.25[a]	1.75
IT vs SPC	7	0.44	1.90	7	2.88[a]	1.90
IT vs T	16	0.73	1.75	13	3.02[a]	1.77
IT vs ME	5	1.85	2.02	6	3.13[a]	1.94
SPC vs T	21	0.43	1.72	16	2.69[a]	1.75
SPC vs ME	10	1.92[a]	1.81	9	0.41	1.83
T vs ME	19	0.96	1.73	15	2.60[a]	1.75
<60 DOC vs >60 DOC	36	1.03	1.70	35	1.92[a]	1.70

Continued...

Table 5 continuation

Parameter	Water d.f.	t_{cal}	t_{crit}	Soil d.f.	t_{cal}	t_{crit}
6) Sulphate reducing bacteria						
SI vs IT	12	1.67	1.78	14	1.85[a]	1.76
SI vs SPC	14	0.05	1.76	17	2.61[a]	1.74
SI vs T	25	1.87[a]	1.71	22	2.02[a]	1.72
SI vs ME	13	0.42	1.77	16	1.72	1.75
IT vs SPC	6	1.83	1.94	7	0.32	1.90
IT vs T	17	2.75[a]	1.74	14	0.55	1.76
IT vs ME	5	1.50	2.02	6	0.84	1.94
SPC vs T	21	1.74	1.72	17	0.89	1.74
SPC vs ME	9	0.35	1.83	9	0.75	1.83
T vs ME	20	1.00	1.73	16	1.84[a]	1.75
<60 DOC vs >60 DOC	38	0.26	1.70	37	1.27	1.70

Parameter	Water d.f.	t_{cal}	t_{crit}
7) Ammonia			
SI vs SPC	11	1.33	1.80
SI vs T	5	0.02	2.02
SI vs T	21	2.10[a]	1.72
SI vs ME	9	0.65	1.83
IT vs SPC	6	4.83[a]	1.94
IT vs T	16	12.56[a]	1.75
SPC vs T	21	28.00[a]	1.72
IT vs ME	5	6.90[a]	2.02
T vs ME	18	0.13	1.73
SPC vs ME	10	0.15	1.81
<60 DOC vs >60 DOC	3	1.68	2.35

Continued...

Table 5 continuation

8) Nitrite			
SI vs IT	4	1.05	2.13
SI vs SPC	9	1.95 [a]	1.83
SI vs ME	8	0.96	1.86
IT vs SPC	5	0.60	2.02
SPC vs ME	9	0.76	1.83
SPC vs T	22	1.30	1.72
<60 DOC vs >60 DOC	3	6.17 [a]	2.35
9) Nitrate			
T vs SPC	16	2.65 [a]	1.75
T vs IT	16	1.12	1.75
T vs ME	18	1.85 [a]	1.73
T vs SI	18	1.73 [a]	1.73
SPC vs SI	10	2.03 [a]	1.81
SPC vs ME	10	0.66	1.81
IT vs SPC	6	0.50	1.94
IT vs Si	4	1.42	2.13
SI vs ME	8	0.33	1.86
<60 DOC vs >60 DOC	3	0.10	2.35

a = P<0.05

Table 6. Percentage change in nutrient concentration in microcosm experiment.

NH_3 removal rate

Treatments \ Ammonia level	1 ppm	2 ppm	3 ppm	4 ppm
Bacillus sp	92.3	95.2	88.3	89.4
Nitrosomonas sp + *Nitrobacter* sp	91.2	95.7	86.5	85.8
Nitrosomonas sp + *Bacillus* sp+ Microcon	97.5	98.6	99.2	98.1
Nitrosomonas sp + *Bacillus* sp	95.9	98.9	98.5	97.9
Biocult	88.6	89.9	80.5	78.0
Epicin	86.6	84.0	81.4	60.7
Control	49.4	37.5	28.2	35.3

NO_2 increase rate

Treatments \ Ammonia level	1 ppm	2 ppm	3 ppm	4 ppm
Bacillus sp	99.0	98.9	91.3	92.6
Nitrosomonas sp + *Nitrobacter* sp	98.5	95.8	89.5	93.6
Nitrosomonas sp + *Bacillus* sp+ Microcon	-86.9	80.1	-10.0	94.7
Nitrosomonas sp + *Bacillus* sp	99.9	92.0	98.1	99.9
Biocult	55.1	63.3	60.0	63.4
Epicin	47.6	41.4	62.6	44.8
Control	75.5	51.6	58.8	50.2

NO_3 increase rate

Treatments \ Ammonia level	1 ppm	2 ppm	3 ppm	4 ppm
Bacillus sp	11.1	0.02	3.0	-23.5
Nitrosomonas sp + *Nitrobacter* sp	17.6	22.5	-14.7	16.3
Nitrosomonas sp + *Bacillus* sp+ Microcon	2.0	8.0	-5.2	-8.1
Nitrosomonas sp + *Bacillus* sp	4.0	2.0	-2.7	-20.4
Biocult	62.8	70.5	60.5	53.4
Epicin	65.1	46.2	62.0	47.0
Control	-27.6	-61.4	-42.5	-49.6

Table 7. Percentage increase in nutrient concentration in simulated pond condition.

Treatment	NH_3	NO_2	NO_3
Biocult	42.32	26.25	79.65
Epicin	45.85	58.12	76.41
NB – Mix	42.29	44.76	49.42
Control	43.88	55.74	63.64

Table 8. F – values computed for different treatments.

Parameter	Fcal	Fcrit	Level of significance	Critical difference
NH_3 – 1 ppm				
NH_3	1.249	2.29	P>0.05	
NO_2	10.587	2.29	P<0.05	101.5
NO_3	5.46	2.29	P<0.05	7.2
NH_3 – 2 ppm				
NH_3	2.388	2.29	P<0.05	5588
NO_2	9.69	2.29	P<0.05	115.2
NO_3	4.988	2.29	P<0.05	6.71
NH_3 – 3 ppm				
NH_3	2.34	2.29	P<0.05	7546
NO_2	3.76	2.29	P<0.05	151.2
NO_3	4.919	2.29	P<0.05	6.23
NH_3 – 4 ppm				
NH_3	1.94	2.29	P>0.05	
NO_2	5.716	2.29	P<0.05	161.8
NO_3	3.868	2.29	P<0.05	6.24
NH_3 – 5 ppm				
NH_3	1.996	2.178	P>0.05	
NO_2	1.43	2.178	P>0.05	
NO_3	2.03	2.178	P>0.05	
***Bacillus* sp**				
NH_3	3.565	2.64	P<0.05	9354.5
NO_2	6.30	2.64	P<0.05	177.1
NO_3	1.27	2.64	P>0.05	
***Nitrosomonas* sp + *Nitrobacter* sp**				
NH_3	5.739	2.64	P<0.05	10226
NO_2	6.247	2.64	P<0.05	183.7
NO_3	0.76	2.64	P>0.05	

Continued...

Table 8 continued

***Bacillus* sp + *Nitrosomonas* sp + Microcon**				
NH_3	2.54	2.94	P>0.05	
NO_2	4.49	2.94	P<0.05	64
NO_3	0.08	2.94	P>0.05	
***Bacillus* sp + *Nitrosomonas* sp +**				
NH_3	3.247	2.94	P<0.05	7900
NO_2	9.48	2.94	P<0.05	130.9
NO_3	0.19	2.94	P>0.05	
Biocult				
NH_3	8.85	2.64	P<0.05	8092.5
NO_2	7.058	2.64	P<0.05	109.5
NO_3	0.618	2.64	P>0.05	
Epicin				
NH_3	19.23	2.64	P<0.05	7376
NO_2	11.22	2.64	P<0.05	109.5
NO_3	1.77	2.64	P>0.05	
Control				
NH_3	57.8	2.64	P<0.05	4314
NO_2	45.58	2.64	P<0.05	81.7
NO_3	3.98	2.64	P<0.05	4.14
Simulated pond condition				
NH_3	0.849	2.94	P>0.05	
NO_2	4.16	2.94	P<0.05	3.16
NO_3	0.15	2.94	P>0.05	

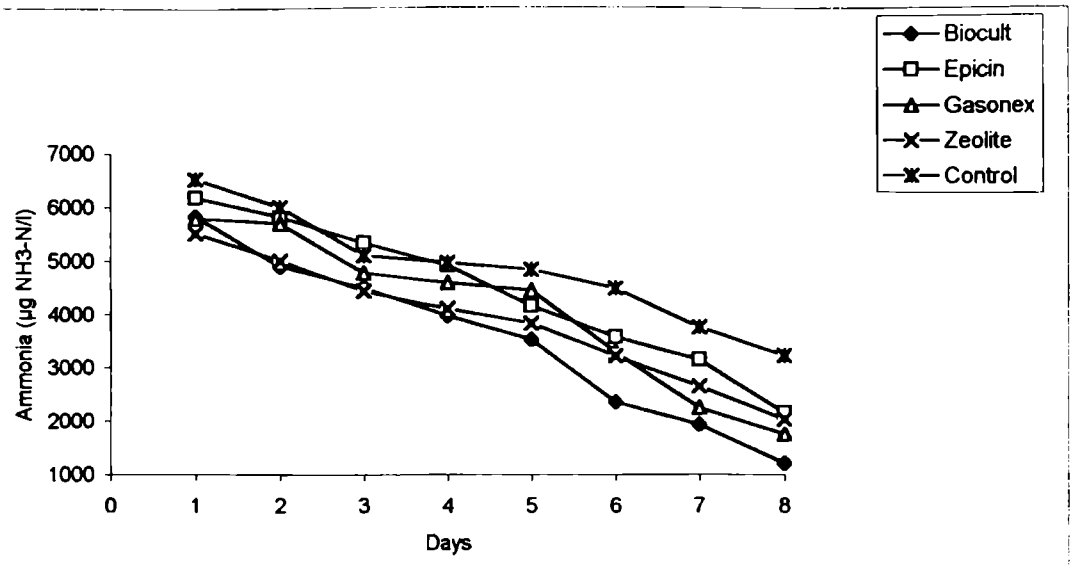


Fig. 2a. Efficacy of commercial bioremediators in removing ammonia (5 ppm) in microcosm experiment

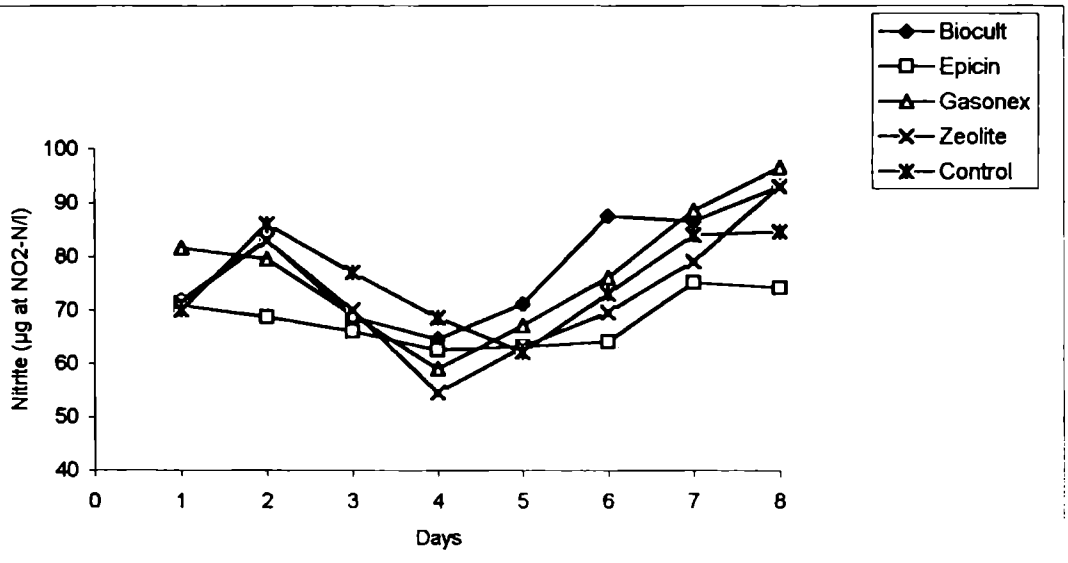


Fig. 2b. Efficacy of commercial bioremediators on nitrite level in microcosm experiment: initial ammonia 5 ppm

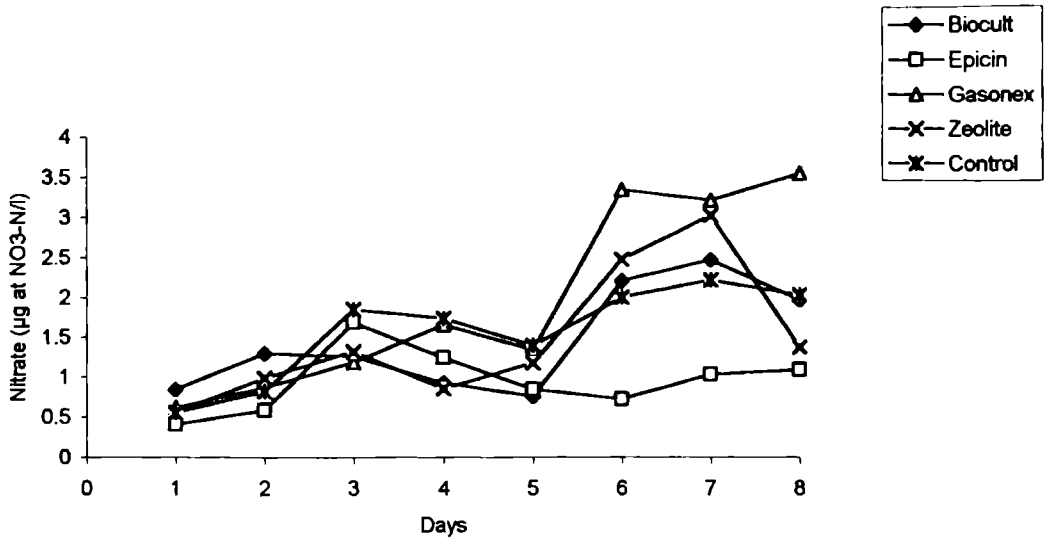


Fig. 2c. Efficacy of commercial bioremediators on nitrate level in microcosm experiment: initial ammonia 5 ppm

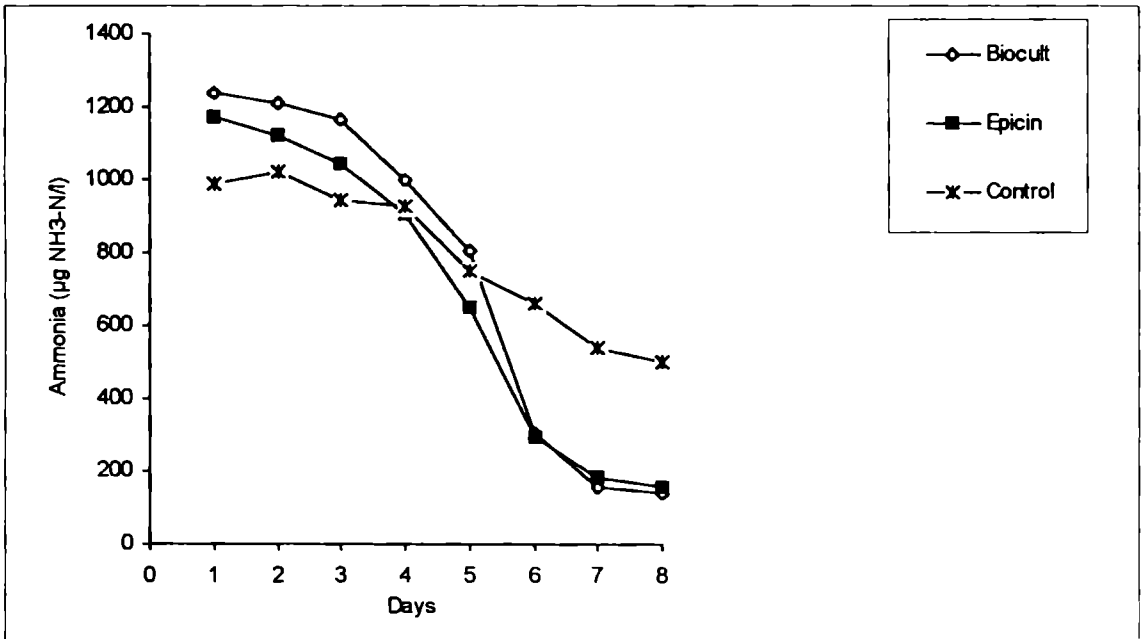


Fig. 3a. Efficacy of commercial bioremediators in removing ammonia (1 ppm) in microcosm experiment

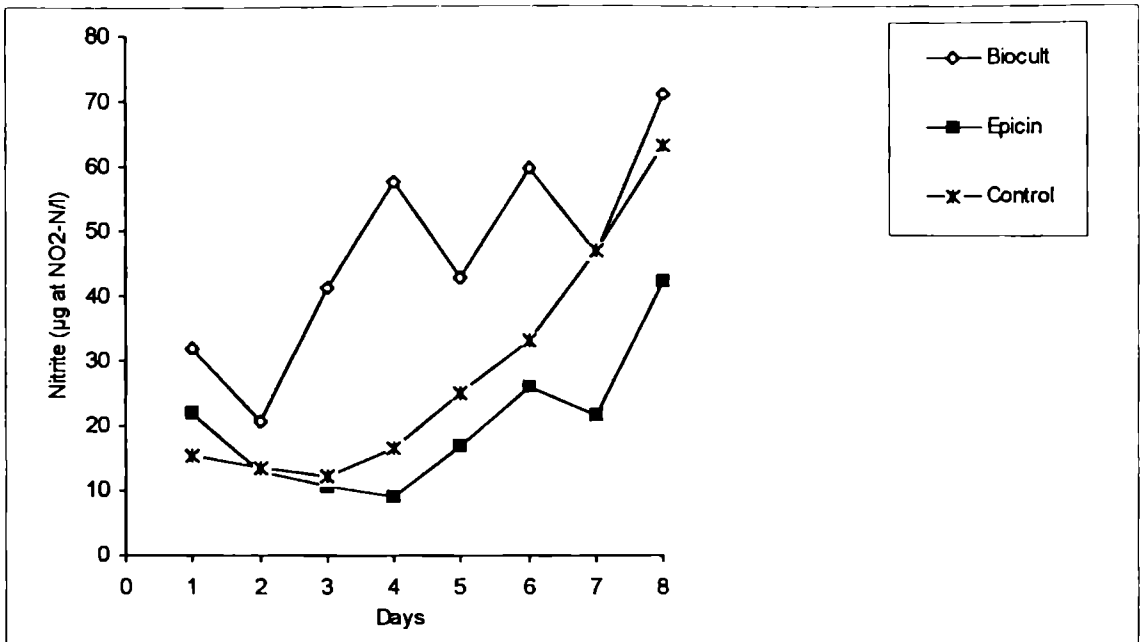


Fig. 3b. Efficacy of commercial bioremediators on nitrite level in microcosm experiment: initial ammonia 1 ppm

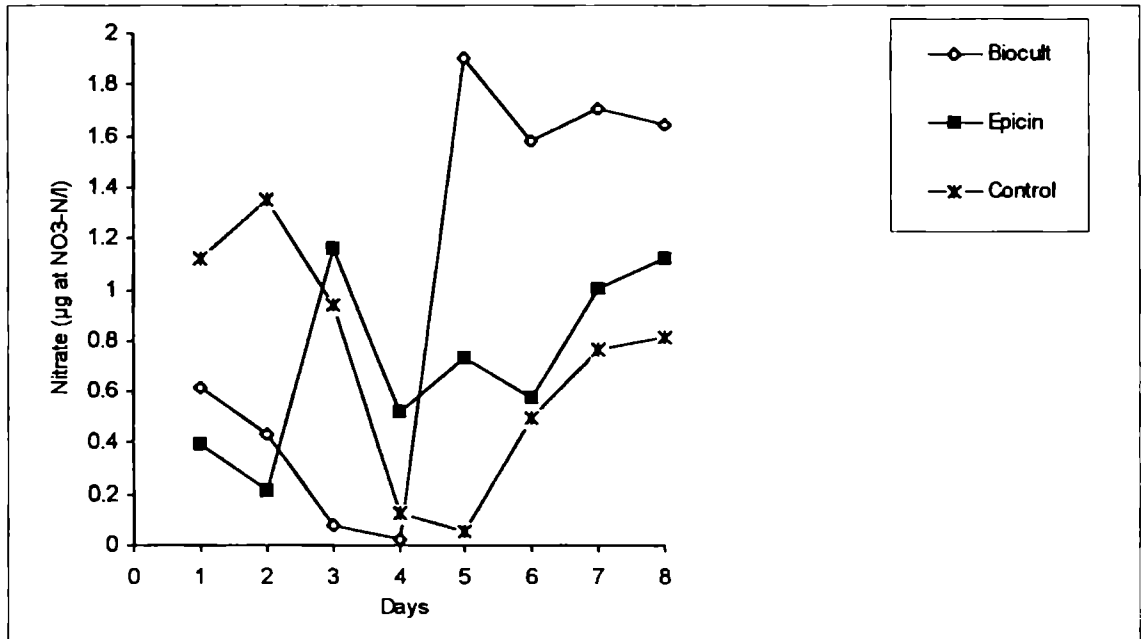


Fig. 3c. Efficacy of commercial bioremediators on nitrate level in microcosm experiment: initial ammonia 1 ppm

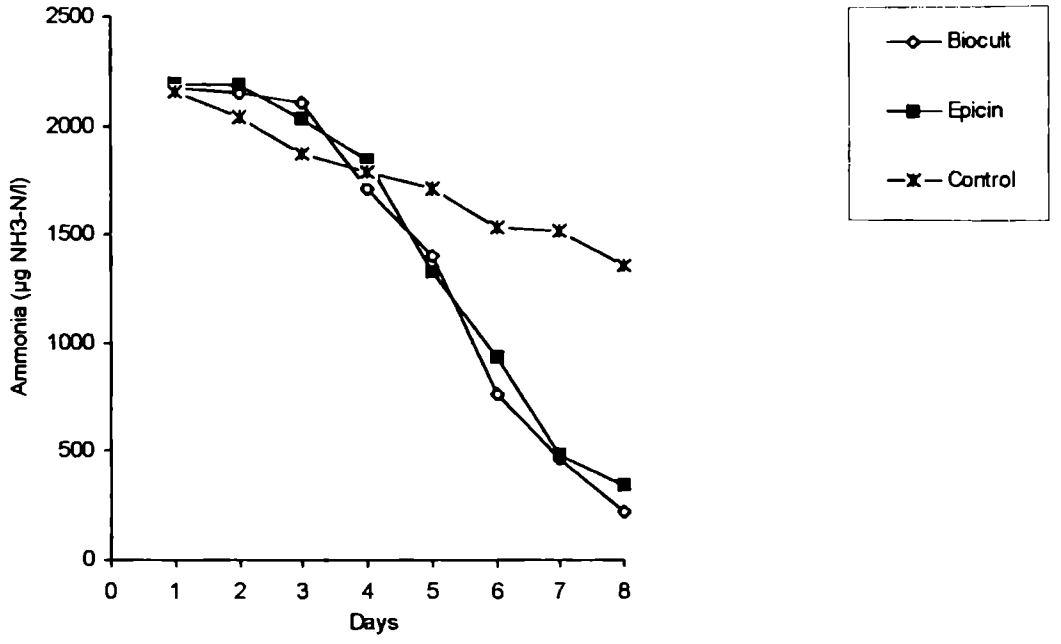


Fig. 4a. Efficacy of commercial bioremediators in removing ammonia (2 ppm) in microcosm experiment

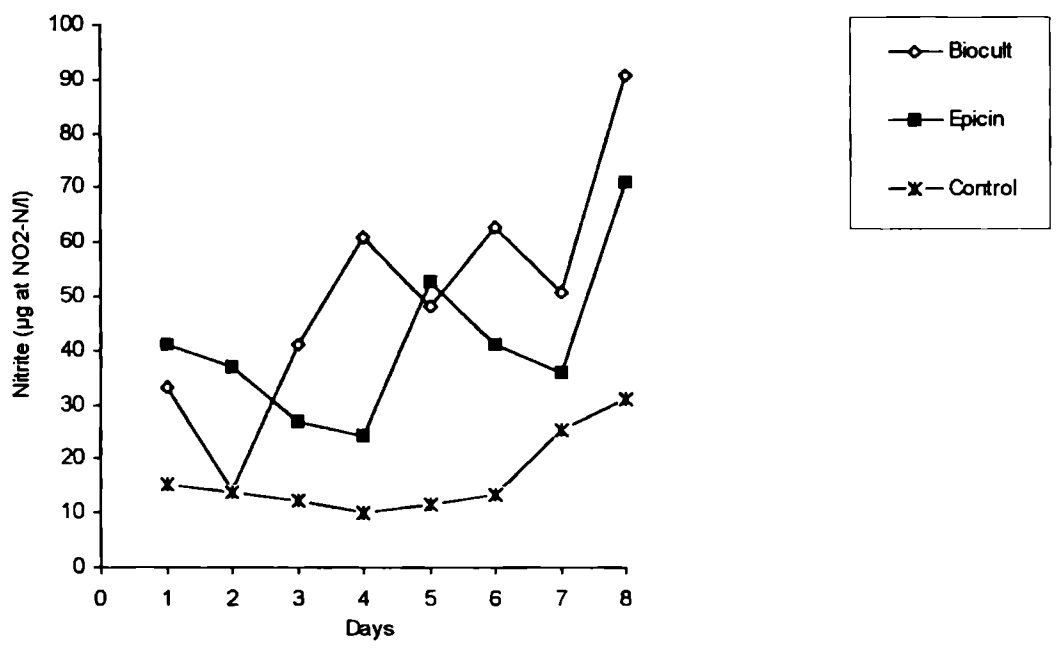


Fig. 4b. Efficacy of commercial bioremediators on nitrite level in microcosm experiment: initial ammonia 2 ppm

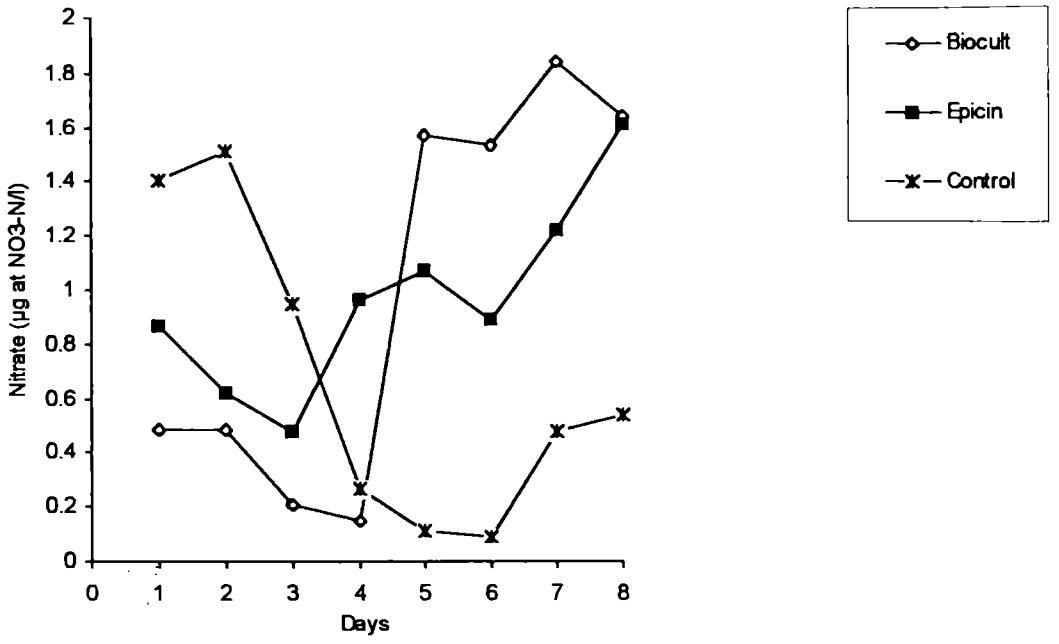


Fig. 4c. Efficacy of commercial bioremediators on nitrate level in microcosm experiment: initial ammonia 2 ppm

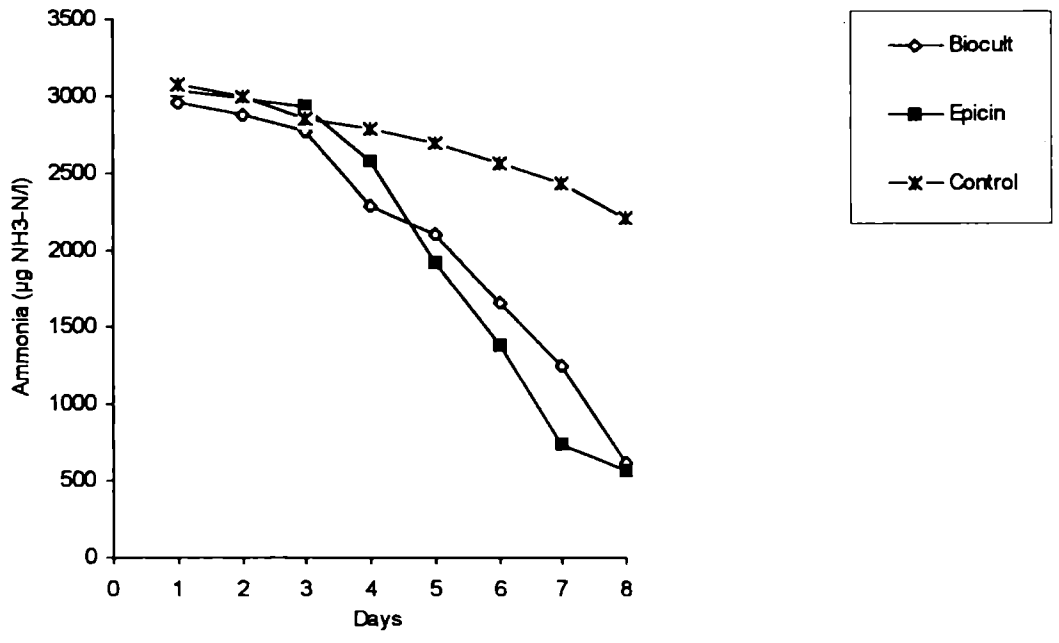


Fig. 5a. Efficacy of commercial bioremediators in removing ammonia (3 ppm) in microcosm experiment

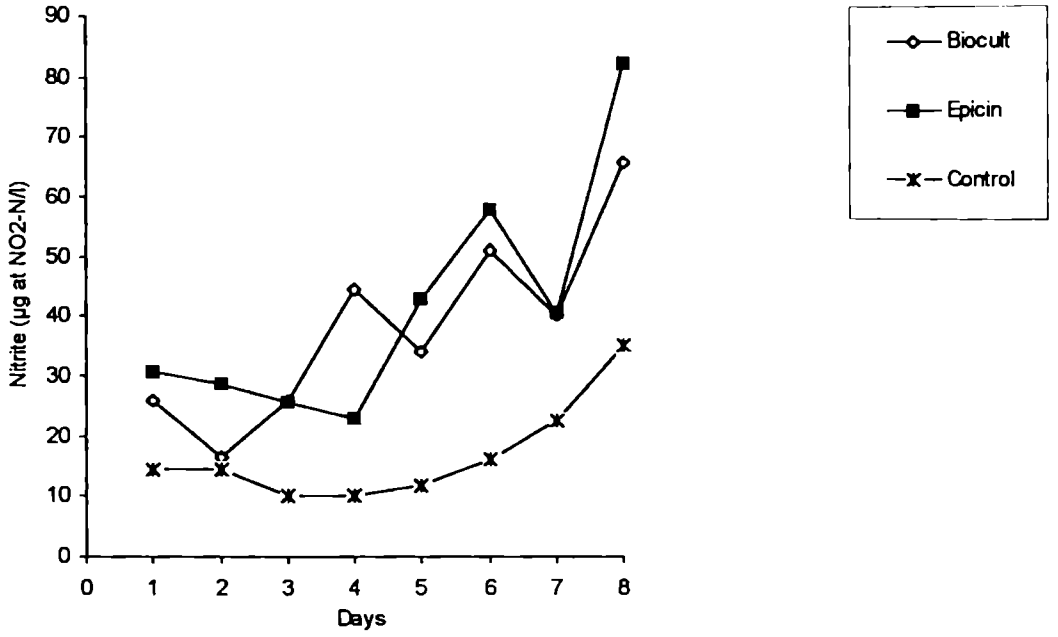


Fig. 5b. Efficacy of commercial bioremediators on nitrite level in microcosm experiment: initial ammonia 3 ppm

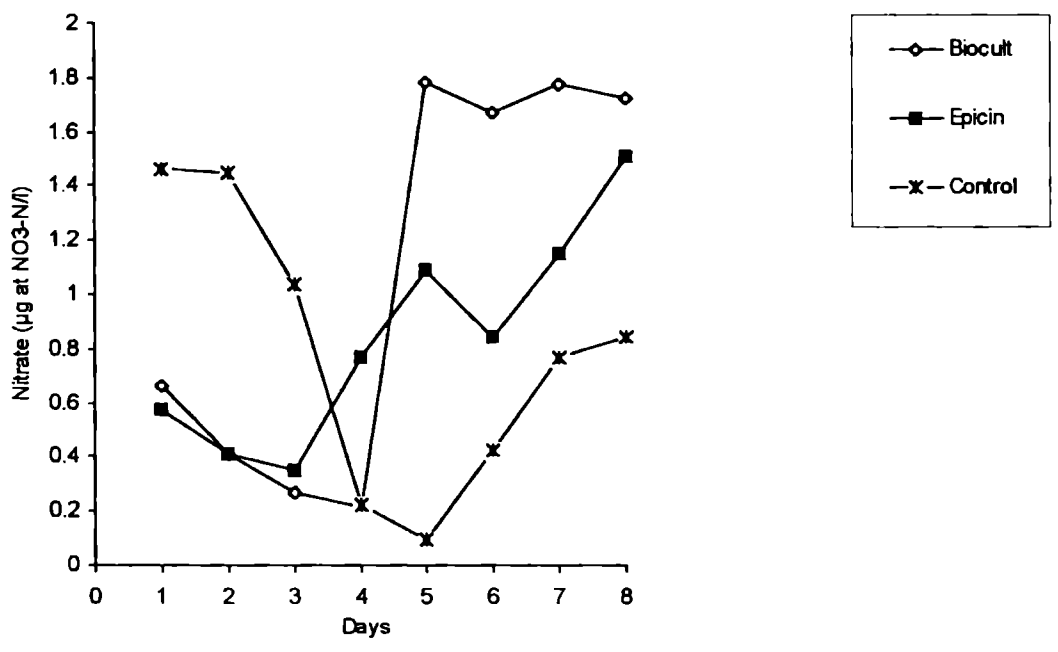


Fig. 5c. Efficacy of commercial bioremediators on nitrate level in microcosm experiment: initial ammonia 3 ppm

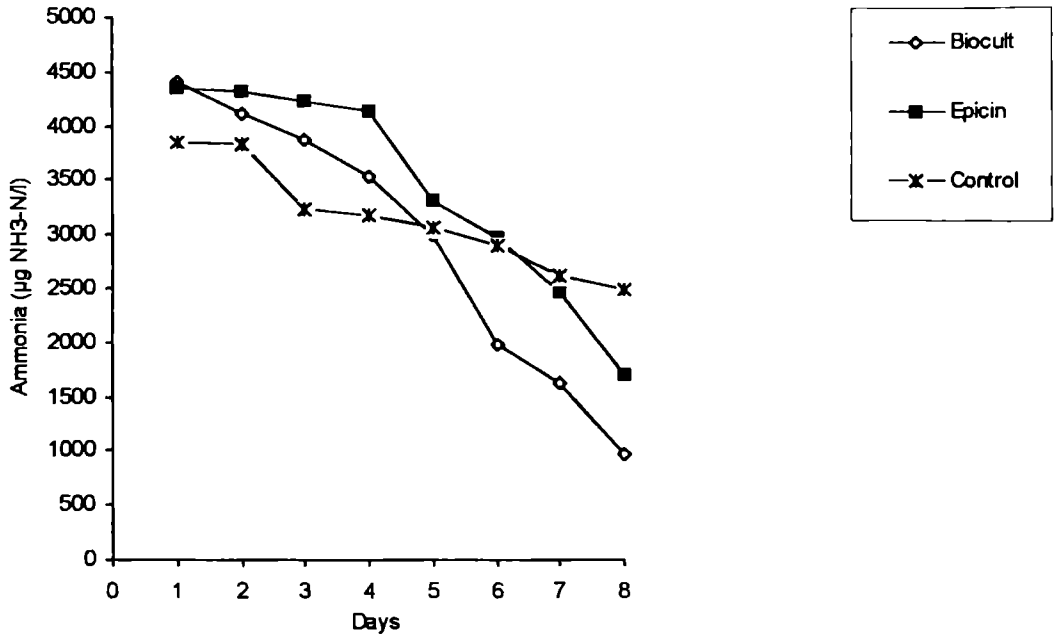


Fig. 6a. Efficacy of commercial bioremediators in removing ammonia (4 ppm) in microcosm experiment

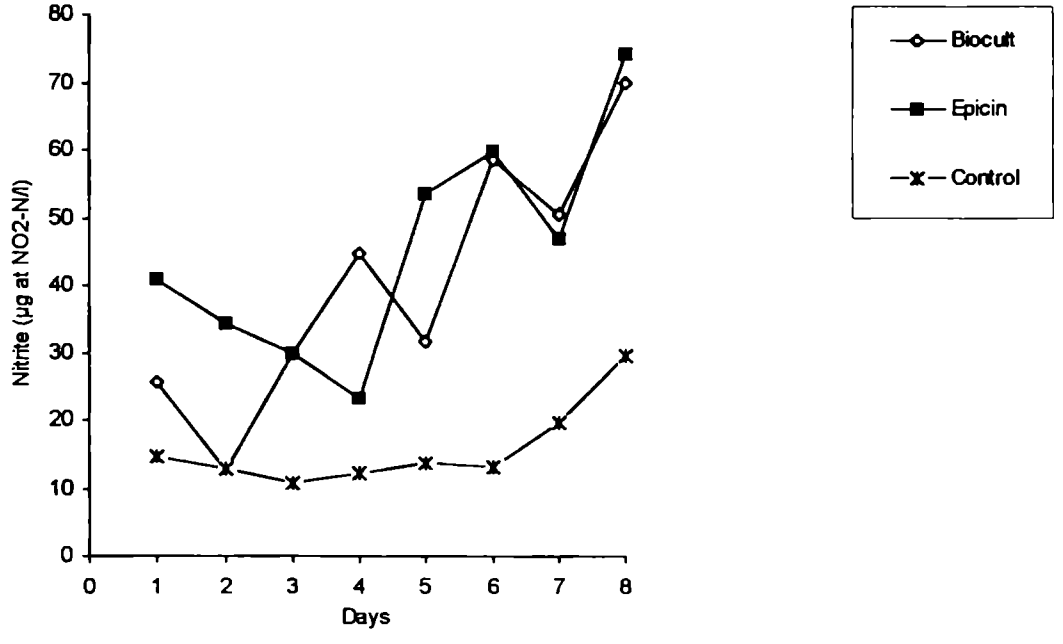


Fig. 6b. Efficacy of commercial bioremediators on nitrite level in microcosm experiment: initial ammonia 4 ppm

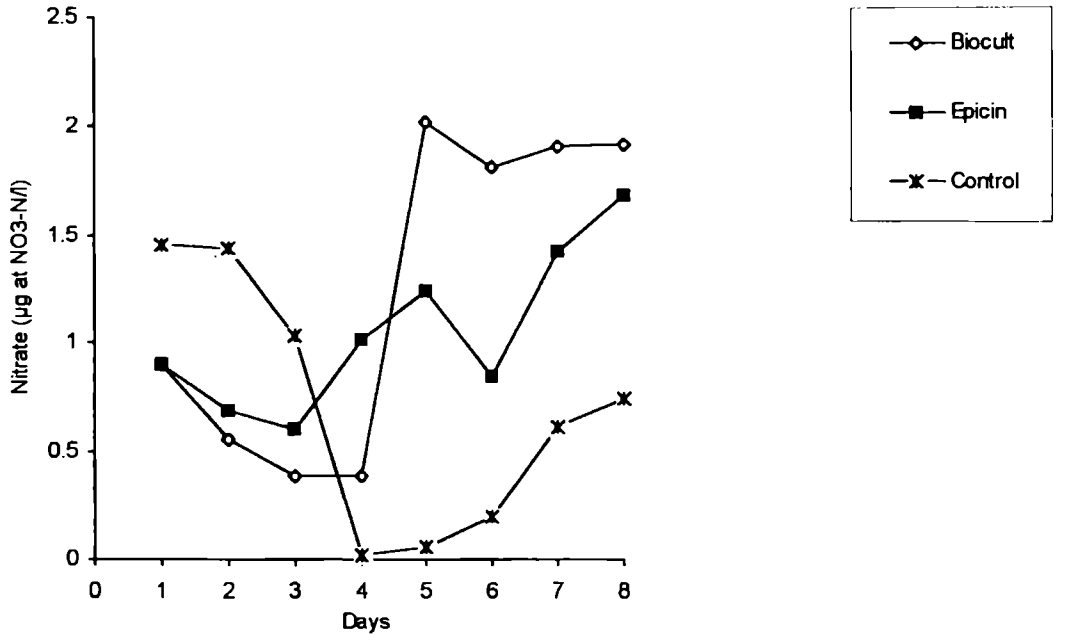


Fig. 6c. Efficacy of commercial bioremediators on nitrate level in microcosm experiment: initial ammonia 4 ppm

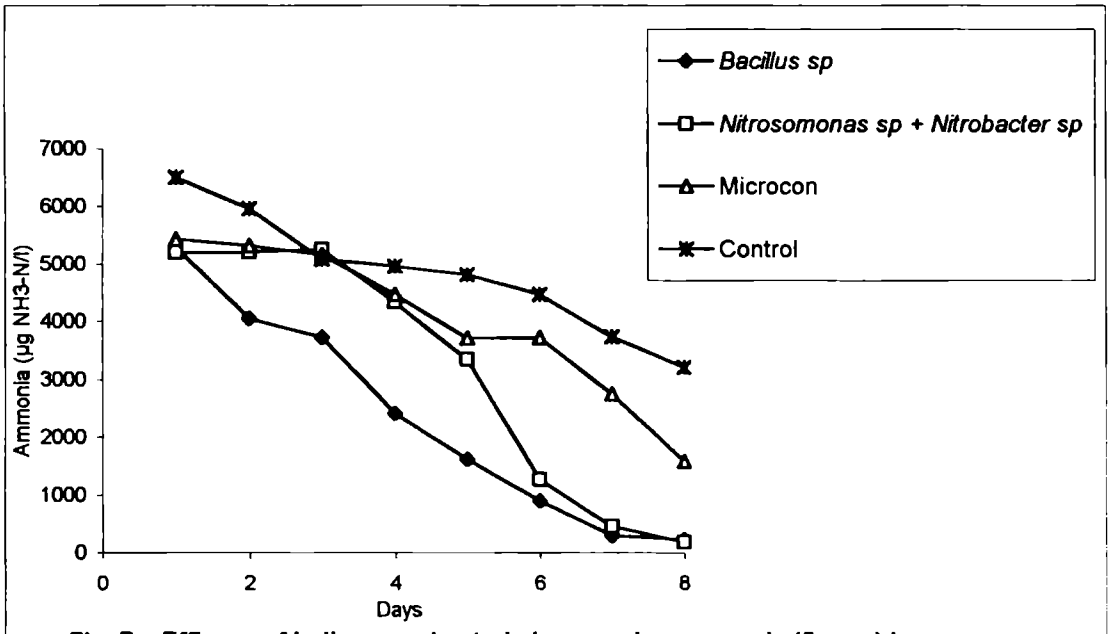


Fig. 7a. Efficacy of indigenous bacteria in removing ammonia (5 ppm) in microcosm experimnet

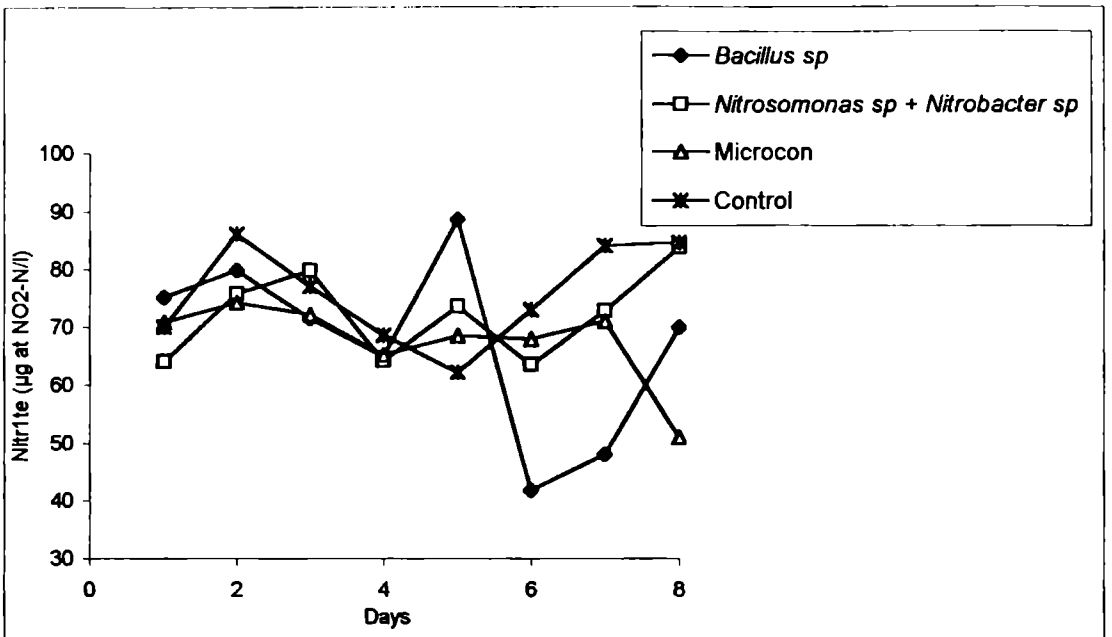


Fig. 7b. Efficacy of indigenous bacteria on nitrite level in microcosm experimnet: initial ammonia 5 ppm

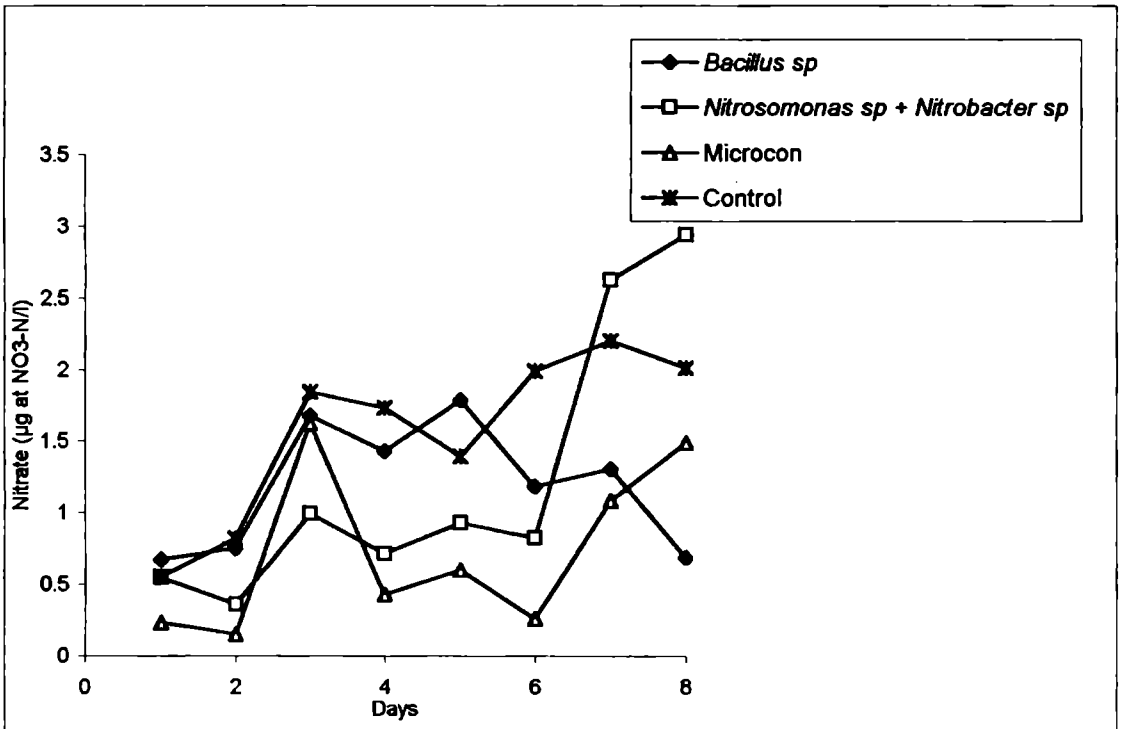


Fig. 7c. Efficacy of indigenous bacteria on nitrate level in microcosm experimnet: initial ammonia 5 ppm

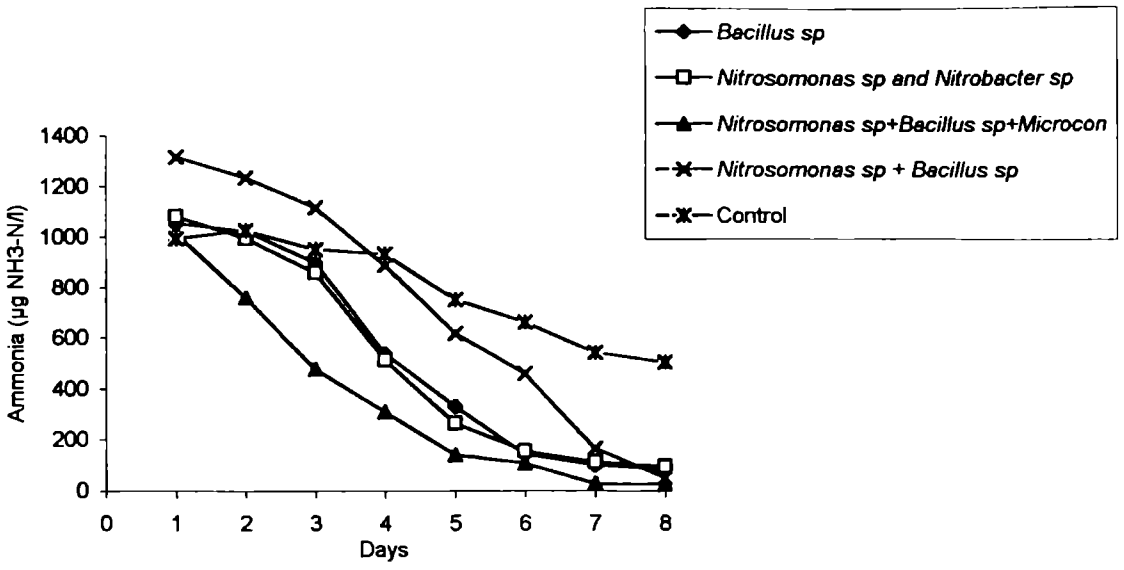


Fig. 8a. Efficacy of indigenous bacteria in removing ammonia (1 ppm) in microcosm experiment

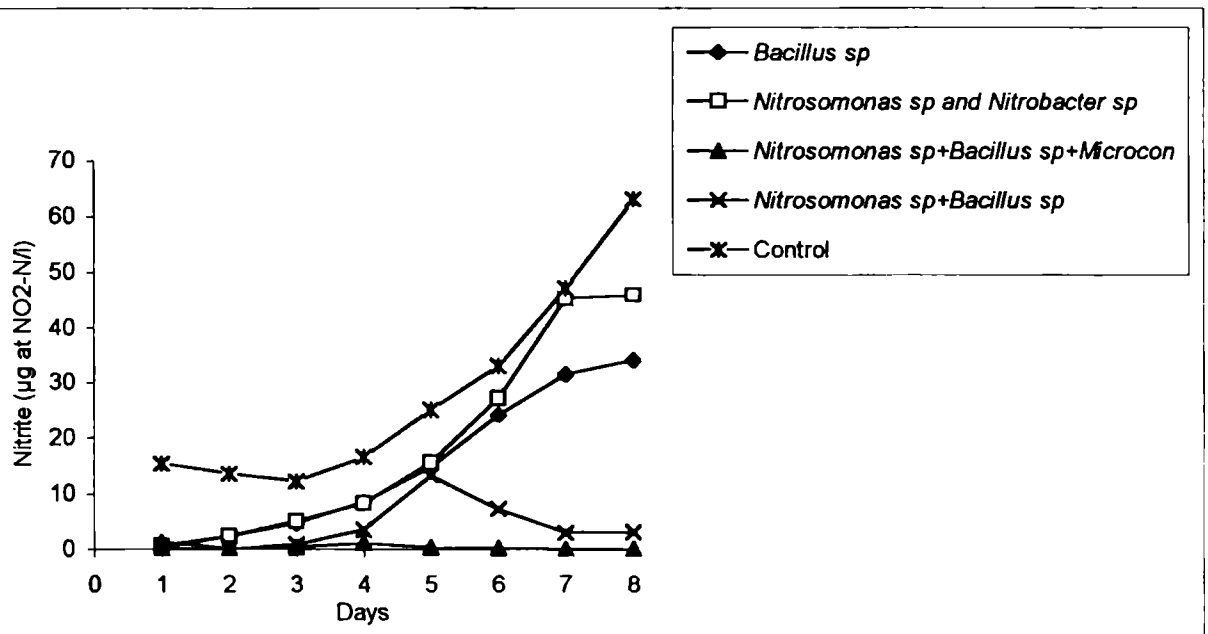


Fig. 8b. Efficacy of indigenous bacteria on nitrite level in microcosm experiment: initial ammonia 1 ppm

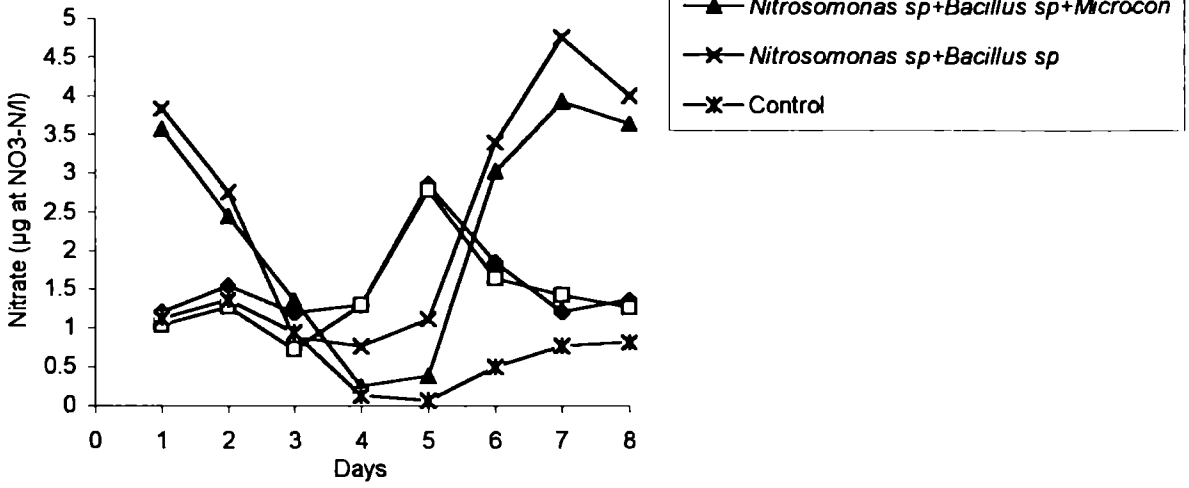


Fig. 8c. Efficacy of indigenous bacteria on nitrate level in microcosm experiment: initial ammonia 1 ppm

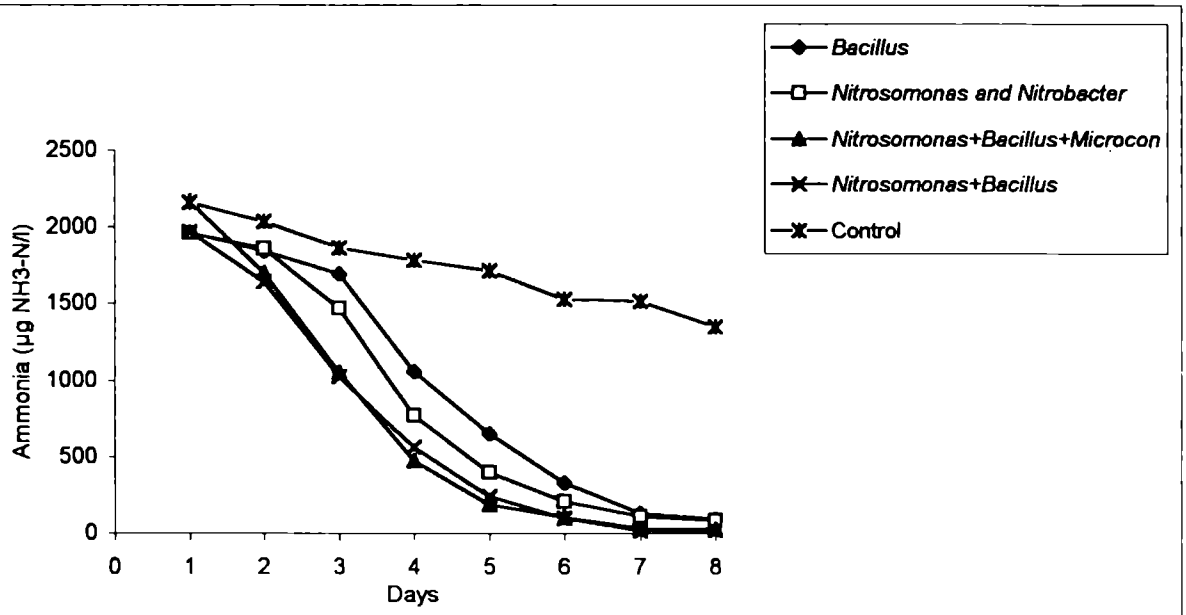


Fig. 9a. Efficacy of indigenous bacteria in removing ammonia (2 ppm) in microcosm experiment

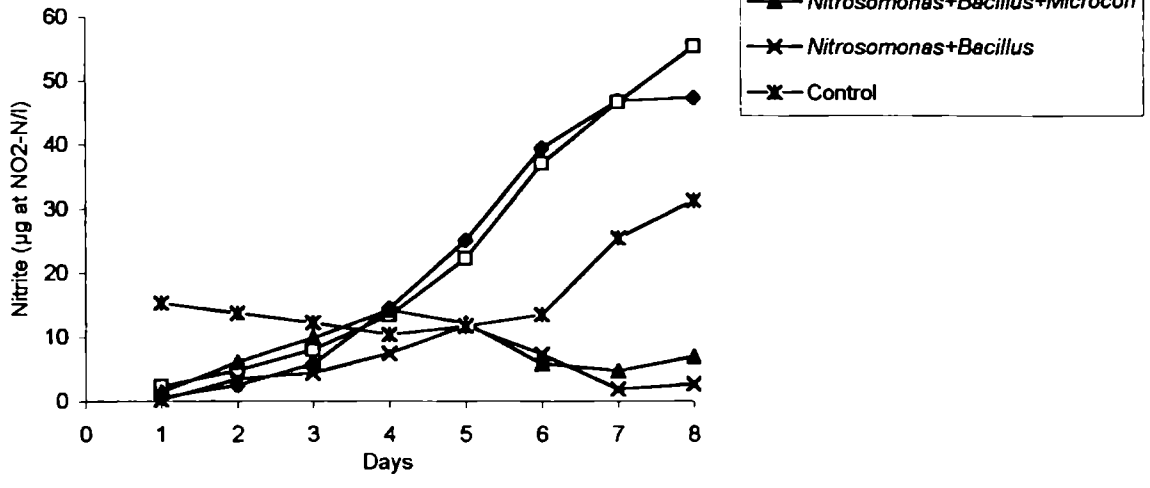


Fig. 9b. Efficacy of indigenous bacteria on nitrite level in microcosm experiment: initial ammonia 2 ppm

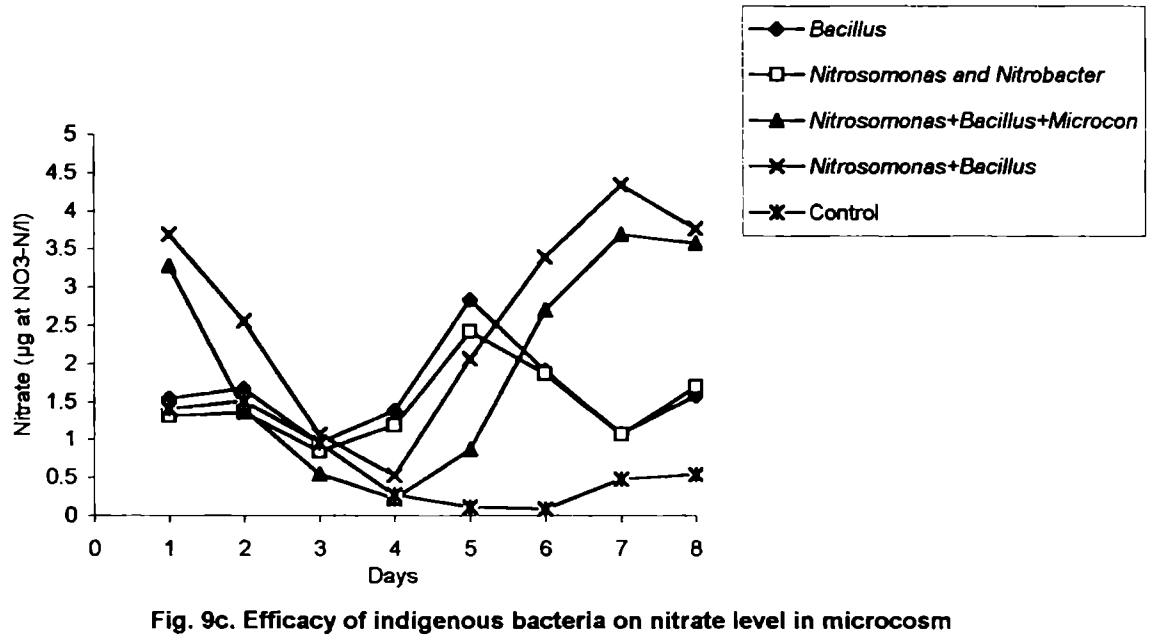


Fig. 9c. Efficacy of indigenous bacteria on nitrate level in microcosm experiment: initial ammonia 2 ppm

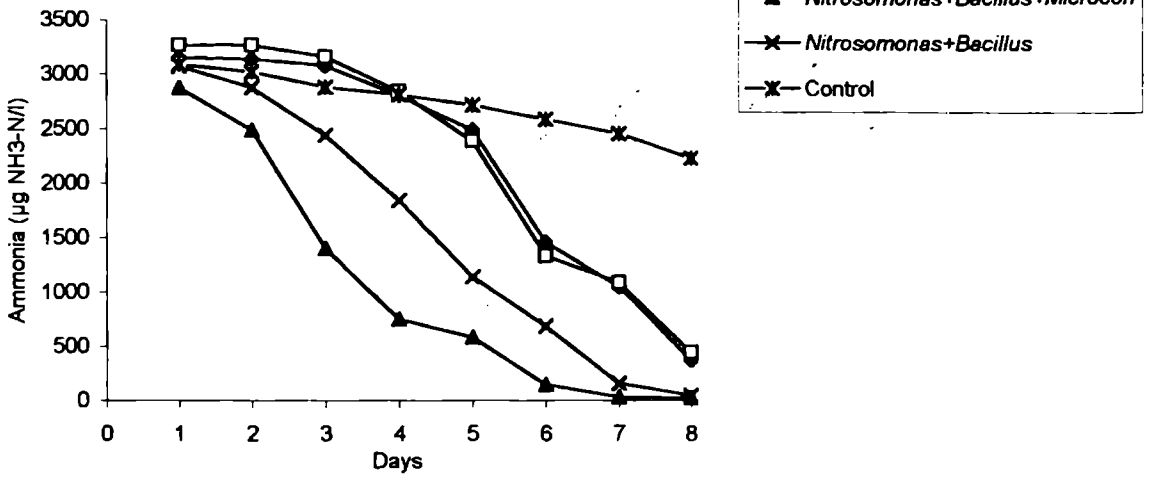


Fig. 10a. Efficacy of indigenous bacteria in removing ammonia in microcosm experiment

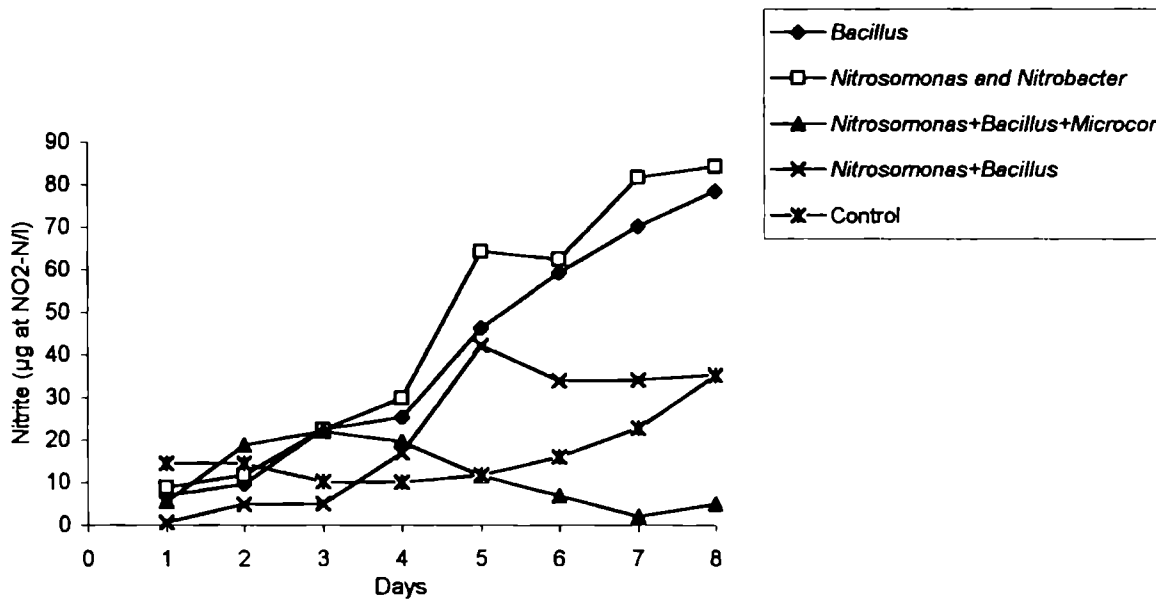


Fig. 10b. Efficacy of indigenous bacteria on nitrite level in microcosm experiment: initial ammonia 3 ppm

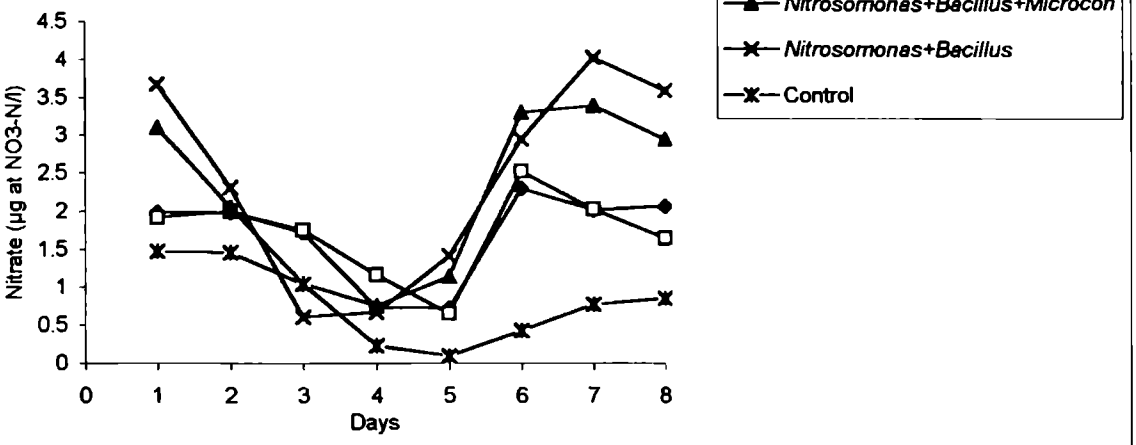


Fig. 10c. Efficacy of indigenous bacteria on nitrate level in microcosm experiment: initial ammonia 3 ppm

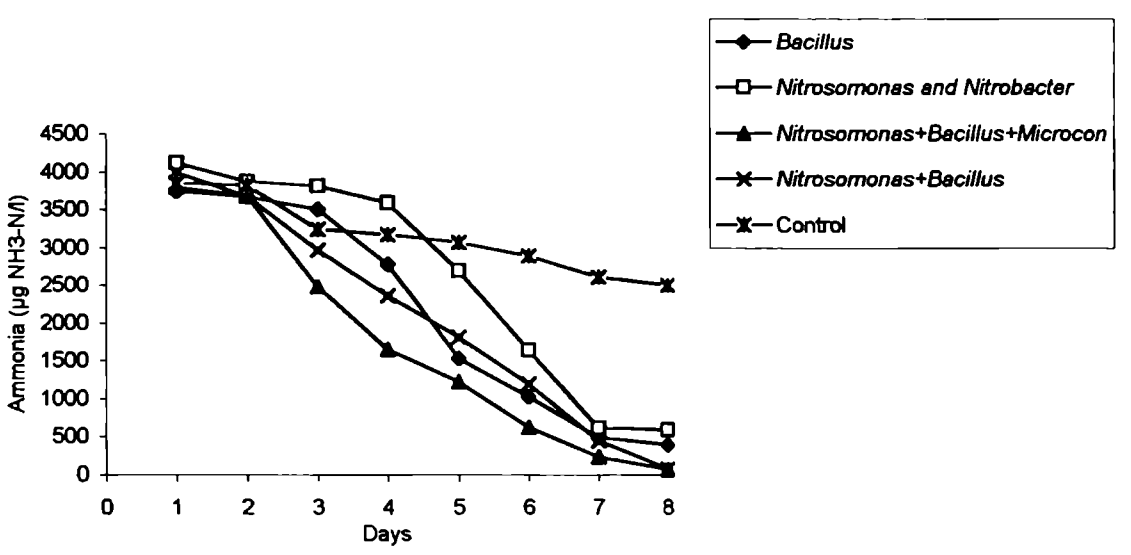


Fig. 11a. Efficacy of indigenous bacteria in removing ammonia (4 ppm) in microcosm experiment

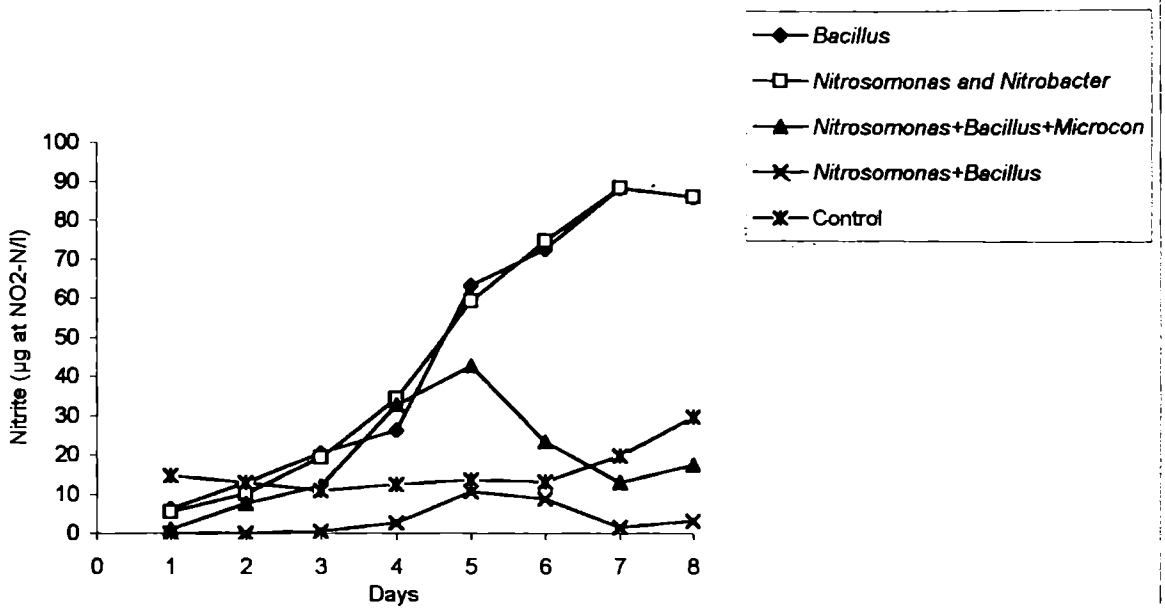


Fig. 11b. Efficacy of indigenous bacteria on nitrite level in microcosm experiment: initial ammonia 4 ppm

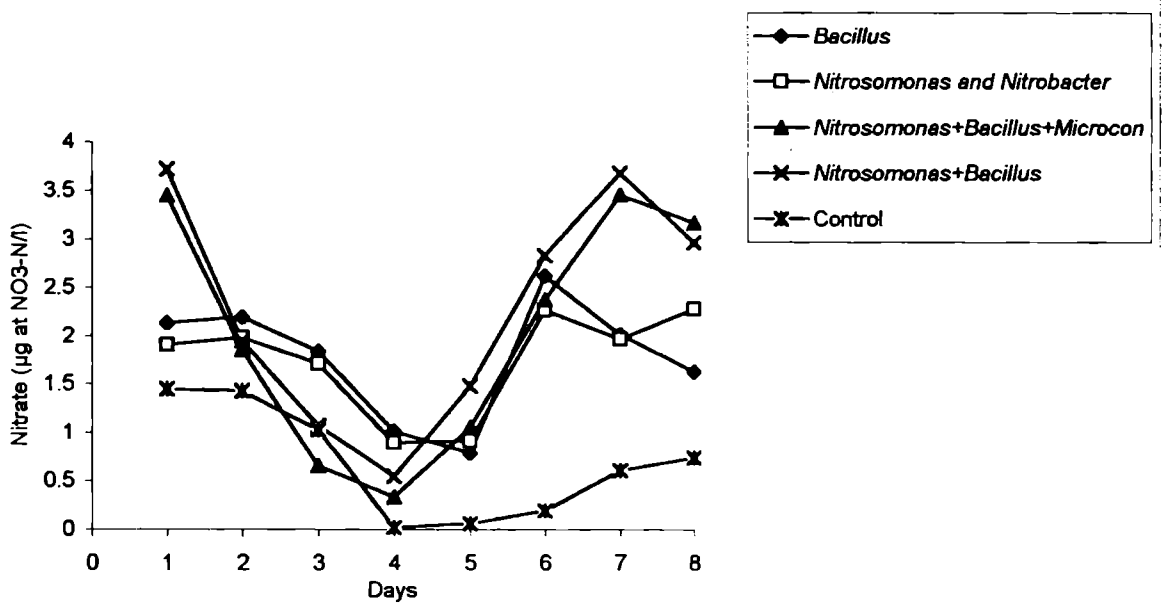


Fig. 11c. Efficacy of indigenous bacteria on nitrate level in microcosm experiment: initial ammonia 4 ppm

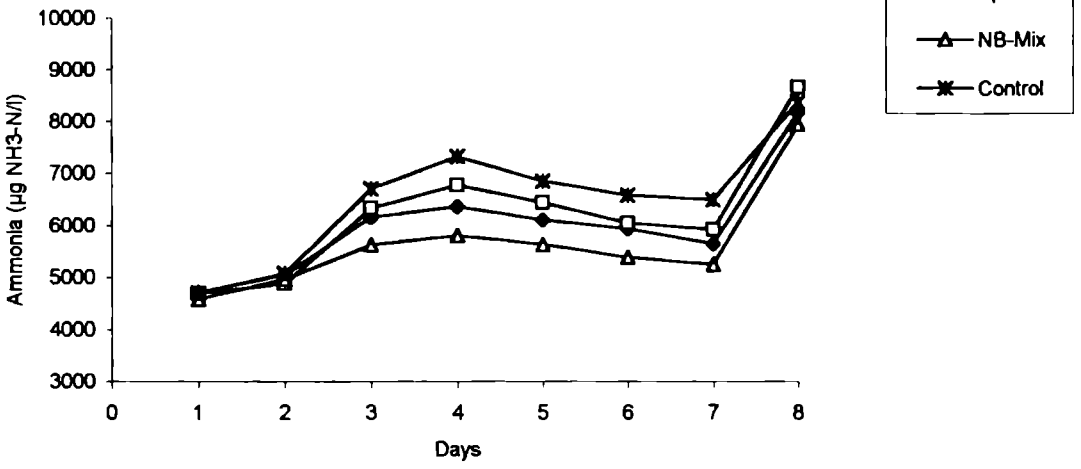


Fig. 12a. Efficacy of bioremediators on ammonia level in simulated pond condition

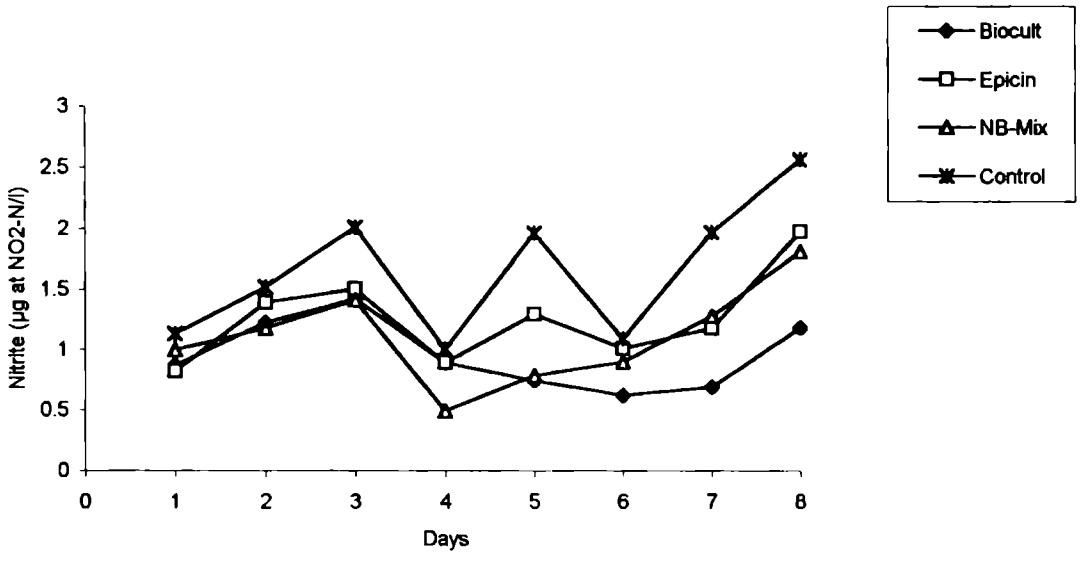


Fig. 12b. Efficacy of bioremediators on nitrite level in simulated pond condition

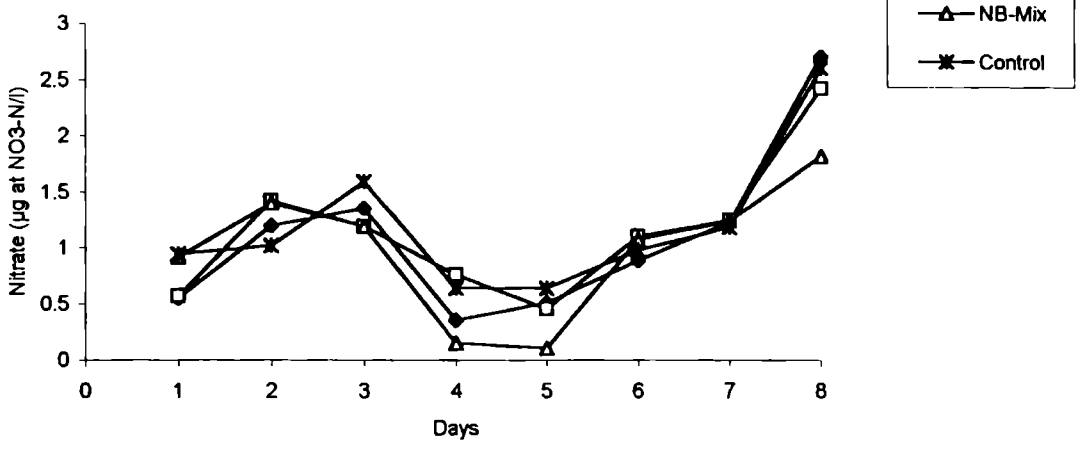


Fig. 12c. Efficacy of bioremediators on nitrate level in simulated pond condition

Chapter - 5

DISCUSSION

5. DISCUSSION

Water quality and disease control are interdependent and linked to the microbial activities in aquaculture system. Microbial processes affect water quality factors such as the levels of dissolved oxygen, NH_3, NO_2^- and sulphide (Moriarty, 1996). One of the most important factors affecting the shrimp production is the build up and toxicity of NH_3 with the intensification of shrimp culture. As with many other industries, the intensive/rapid growth in shrimp aquaculture has brought with it the problem of environmental pollution. Shrimp culture all over the world has been frequently affected by the viral and bacterial diseases (Johnson, 1990; Lightner, 1993a, b; Mohanty and Sunderray, 1999). To combat diseases, a host of aquaculture drugs are used (Karunasagar and Karunasagar, 1996; Burman, 2002) possibly creating environmental hazards. Thus, research and development to improve the ecological environment of aquaculture has become the focus of attention of international aquaculture.

West Bengal, one of the maritime States of India, is bestowed with vast potential of natural resources for brackish water shrimp production. Shrimp culture in the State is being carried out by traditional, improved traditional, modified extensive, stagnant pond culture and semi-intensive methods (Burman, 2002). Distribution of total heterotrophic bacteria, luminous bacteria and presumptive vibrios in these aquaculture systems of West Bengal have been studied and described (Sengupta, 2002; Kamilya, 2002). However, studies on the distribution of aquatic bacteria with varied physiological characteristics are lacking, as these are very much necessary to understand the microbial activities in aquaculture system. Such studies are essential to manipulate and improve the ecological environment of microbes in aquaculture.

This study was, therefore, undertaken with an aim to monitor the bacteria of different physiological characteristics in shrimp culture systems of West Bengal and to develop bioremedial measures for use in shrimp aquaculture.

5.1. Monitoring of bacteria of varied physiological characteristics in shrimp culture ponds

5.1.1. Total heterotrophic bacteria

As seen in Tables 2 and 3, total heterotrophic bacterial counts in the range of $1.10x10^2 - 1.02x10^7$/ml were recorded in shrimp culture systems with a mean of always above 10^6/ml of pond water except the traditional system. The results revealed the abundant availability of nutrients derived from excess feed, shrimp excreta and other dead and decaying organic matter for bacterial growth in shrimp culture systems of West Bengal. The levels of total heterotrophs recorded in the present study are in agreement with earlier reports (Peranginangin *et al.*, 1992; PremAnand *et al.*, 1996; Sharmila *et al.*, 1996; Sung *et al.*, 2001). The observed significant differences in the total heterotrophs ($P<0.05$) between the traditional and other systems revealed that the management practices influence the bacterial populations of shrimp culture systems. Barat and Jana (1987) studied the effect of farm management on the distribution pattern of protein mineralizing and ammonifying bacterial population in fish culture tanks. They reported high levels of protein mineralizing and ammonifying bacteria in catfish ponds than in carp culture ponds. Jana and Roy (1983) observed differences in qualitative microbial population in traditional, mono and polyculture systems and were related to the fish culturing practices adopted. The results of the present study are in conformity with Moriarty (1986) who reported higher heterotrophic bacterial population in the water and sediment samples of ponds receiving organic matter as input from feed. However, the differences in the total heterotrophs in other systems were insignificant ($P>0.05$), so also in earlier studies from West Bengal (Kamilya, 2002; Sengupta, 2002).

Further, the increasing levels of NH_3 in the semi-intensive system and to some extent in other systems was coincided with the increasing levels of total heterotrophs; while in traditional system, the low levels of heterotrophs correlated with low levels of NH_3 (Tables 2, 3 and 5). However, no significant correlation between NH_3 and total heterotrophs was observed in any of the systems as the number of samples analysed was too low ($r = -0.83$; $df = 4$; $P>0.05$). It is known that the NH_3 within the shrimp pond is largely derived from the breakdown of protein added to ponds in the form of shrimp feed and subsequent excretion by the shrimp.

The total heterotrophs recorded in shrimp pond sediment samples were higher than in water (Tables 2 and 3), so also in earlier reports (Peranginangin *et al.*, 1992; PremAnand *et al.*, 1996; Sharmila *et. al.*, 1996; Sung *et al.*, 2001; Kamilya, 2002; Sengupta, 2002). The fact is that sediment gives shelter to both planktonic and biofilm forming bacteria, while water column contain only planktonic (free living) bacteria. Besides this, pond sediment provides more nutrients for bacterial growth in comparison to overlying water. Moriarty (1986), however, observed higher counts of heterotrophs in water column than in sediment of a shrimp farm. Further, the mean heterotrophic bacterial counts of semi-intensive shrimp pond sediment recorded the lowest ($2.68\pm1.68x10^6$/g) than other systems, probably due to the dominance of anaerobes and microaerophiles in sediment. The results of this study are in close agreement to Prabhu *et al.* (1999) who recorded heterotrophic bacterial counts in the range of $1.5x10^3 - 4.8x10^7$/ml of pond water and $2.5x10^3 - 1.2x10^8$/g of sediment in shrimp culture ponds of Tamil Nadu. The total heterotrophic bacterial counts of semi-intensive and traditional, stagnant pond culture and traditional, and stagnant pond culture and modified extensive systems were found to differ significantly ($P<0.05$), thus, confirming the influence of management practices on the bacterial population as has been reported earlier (Jana and Roy, 1983; Barat and Jana, 1987; Kamilya, 2002).

5.1.2. *Nitrosomonas* sp

The results presented in Tables 2 and 3 revealed that the counts of *Nitrosomonas* sp were higher in pond sediment than in pond water of all the culture systems. The mean counts of *Nitrosomonas* sp in pond water and pond sediment were higher in traditional ($1x10^4$/100 ml) and modified extensive ($1.9x10^4$/g) systems, respectively. The lowest counts of 0-$3.5x10^2$/100 ml and 2.3 – $1.6x10^4$/g were observed in stagnant pond culture and traditional systems. The counts of *Nitrosomonas* sp of pond water of the present study are quite lower when compared with Bianchi and Bianchi (1982), who reported $1x10^4$/ml of nitrifying bacteria of shrimp pond water. But, the results of the present study are comparatively higher than those reported in Indian shrimp culture ponds (Rao, 1997). He reported low levels of *Nitrosomonas* sp ranging from 0-23/100 ml from shrimp ponds of Karnataka, India.

There existed significant differences in the counts of *Nitrosomonas* sp in pond water samples between semi-intensive and traditional, stagnant pond culture and traditional, and stagnant pond culture and modified extensive systems thereby revealing the influence of culture practices on the counts of *Nitrosomonas* sp.

The counts of *Nitrosomonas* sp of shrimp pond sediment (Tables 2 and 3) from West Bengal were higher than those reported (Rao, 1997) from Karnataka (0-200 /100ml). According to Fry (1987), nitrification is maximal when both O_2 and NH_4^+ are present and so occurs maximally at the oxygenated surface layers of the sediment. Significant differences ($P<0.05$) in the counts of *Nitrosomonas* sp were observed among the culture systems. This probably revealed the influence of culture practices and substrate availability on the counts of *Nitrosomonas* sp in shrimp ponds. Nevertheless, no significant correlation between *Nitrosomonas* sp counts and ammonia ($r = 0.81$; df=4) levels was noted, as the samples analysed were too low. Further, *Nitrosomonas* sp are highly sensitive to sudden changes in temperature, reduction of available nutrients, aquaculture drugs and H_2S (Scott and Gillespie, 1972; Collins *et al.*, 1975; Smith *et al.*, 1981; Joy and

Hollibaugh, 1995). These factors could also have played role in the varying levels of counts of *Nitrosomonas* sp in different systems as many of the West Bengal farmers were reportedly using aquaculture drugs in their farms (Burman, 2002).

5.1.3. *Nitrobacter* sp

The mean counts of *Nitrobacter* sp in pond water, with values always above 10^4/100 ml, were higher than that of *Nitrosomonas* sp in all the culture systems (Tables 2 and 3). Similar results were reported from the shrimp culture ponds of Karnataka, India (Rao, 1997). Significant differences were noted in the *Nitrobacter* sp counts among the pond water samples and also among the pond sediment samples ($P<0.05$) of different culture systems as have been observed with *Nitrosomonas* sp. The correlation between the *Nitrobacter* sp counts and the levels of NO_2 ($r = -0.49$; $df = 4$) was, however, insignificant ($P>0.05$). The results on the observations of bacteria involved in ammonification, nitratation and nitritation in West Bengal shrimp culture systems and also the levels of NH_3, NO_2 and NO_3 well within the safe levels clearly showed that the recycling of nitrogen in shrimp aquaculture is going on actively.

5.1.4. Sulphur oxidizing bacteria (SOB)

The MPN counts of SOB was in the range of 0-3.5x10^4/100 ml, with a peak of MPN 3.5x10^4/100 ml of pond water in improved traditional and semi-intensive systems. The levels in sediments ranged from 0-9.2x10^3/g, with highest values from semi-intensive system (Tables 2 and 3). The SOB were absent in pond water samples of modified extensive and stagnant pond culture systems and pond sediment samples of modified extensive, stagnant pond culture and traditional systems probably due to the non-availability of substrate. The observed counts of SOB in certain shrimp culture systems of West Bengal were quite higher due to the higher availability of sulphur than those reported by Rao (1997) in shrimp culture ponds of Karnataka. He reported counts of SOB in the range of

$2.3x10^1$ - $>2.4x10^3$/100 ml and $2.3x10^2$ - $>2.4x10^4$/100 ml in pond water and pond sediment, respectively. Jorgensen (1983) reported SOB levels of 10^3/ml of water and $10^4 - 10^6$/g of sediment.

The presence of SOB in low numbers or the total absence of SOB in the water and sediment samples of stagnant pond culture and traditional systems could be due to the fact that the activity of SOB is restricted to the H_2S/O_2 interface that exists in the water column and aerobic sediments (Fry, 1987). The SOB counts in pond water of improved traditional system varied significantly ($P<0.05$) with those of traditional system. The counts of SOB in pond water of other systems and in pond sediments, however, differed insignificantly ($P>0.05$). There is little information on the levels of SOB in shrimp culture ponds. However, their presence in the system, although with variations, clearly showed that the recycling of sulphur in shrimp aquaculture system is going on actively.

5.1.5. Sulphur reducing bacteria (SRB)

The MPN counts of SRB were always higher in pond sediment than in pond water samples as they are anaerobic in nature. The ranges of counts of SRB in pond water and pond sediment were 0 – $2.5x10^5$/100 ml and $1.10x10^2$ – $3.48x10^4$/g, respectively (Tables 2 and 3). The counts of SRB recorded in the present study were comparatively higher than those of Rao (1997), who recorded $5.40x10^2$ – $>2.40x10^3$/100 ml of pond water and $5.40x10^3$ – $>2.40x10^4$/100 ml of sediment. Jorgensen (1977a) reported a SRB count of $2.00x10^4 - 1.30x10^5$/cm^2 in sediment. Despite being anaerobes, the SRB were recorded in pond water samples of shrimp farm probably due to the presence of reduced microniche in sediment particles suspended in water. According to Jorgensen (1977b) aerobic heterotrophs consume O_2 in these environment and, thus, keeping the centre of the particle anaerobic and allowing the SRB to flourish.

Further, the SRB counts in pond water of traditional system varied significantly ($P<0.05$) with those of semi-intensive, stagnant pond culture and

improved traditional systems. Significant differences ($P<0.05$) in their counts were also observed in sediment samples between semi-intensive and improved traditional, semi-intensive and stagnant pond culture, semi-intensive and traditional, and traditional and modified extensive systems probably due to the varying levels of availability of substrate for their activity and varying degrees of anaerobiosis. Suplee and Cotner (1996) observed higher counts of SRB in sediments, which have not been dried significantly between crops compared to sediments, which have been dried between crops. This perhaps provides explanation for the differences in the counts of SRB of different shrimp culture systems of West Bengal. This observation also underscores the importance of drying of pond sediments (bottom) between crops.

5.1.6. Nitrate reducing bacteria (NRB)

The MPN counts of NRB were in the range of 0-$7x10^5$/100 ml and 0-$9.2x10^4$/g in pond water and sediment, respectively (Tables 2 and 3). Significant differences ($P<0.05$) in their counts were observed between the pond water samples of modified extensive system with semi-intensive and stagnant pond culture systems. Likewise, in sediment NRB counts also, significant differences ($P<0.05$) were observed between semi-intensive and stagnant pond culture, semi-intensive and modified extensive, improved traditional and stagnant pond culture, improved traditional and traditional, improved traditional and modified extensive, stagnant pond culture and traditional, and modified extensive and traditional systems. This could be due to the differences in the availability of nitrates and organic carbon and also due to the differences in temperature and limiting levels of O_2. The differences ($P<0.05$) in the NRB counts of first 60 DOC in sediment samples with those of later 60 DOC further confirmed the greater availability of substrate and organic carbon for the NRB in sediment and their influence on microbial activities. It has been reported that the denitrification rates are highest in summer and when there is plentiful supply of nitrates and organic carbon

(Wetzel, 1983). According to Herbert (1982), the NRB isolates from estuarine and marine sediments are predominantly fermentative and oxidative bacteria are rare.

5.2. Physico-chemical characteristics of shrimp culture systems

The results presented in Table 4 revealed that all the physico-chemical parameters except salinity were well within the safe levels for culture of shrimps thereby revealing the overall well being of the systems. The water temperature was within the safe levels suggested by MPEDA (1992). The pH values of water were near the optimum value of 7.5-8.7 (MPEDA, 1992). The pH values of sediment samples are in accordance with the values reported by Kamilya (2002) and Sengupta (2002) in the shrimp culture systems of West Bengal. The salinity was too low in the traditional system (Malancha and Khariberia), since the source of water was from River Bidyadhari and its tributaries which had salinity levels of 5-10 ppt. The range of salinity in other type of systems were sometimes low compared to the safe levels (MPEDA, 1992) due to drop of salinity in source water as a result of rainfall and runoff.

The NH_3-N levels in all the systems were significantly different ($P<0.05$) and far less than the safe level (<1 ppm) suggested by MPEDA (1992). The results are in accordance with the values reported by Briggs and Funge-Smith (1994), i.e., 0.2-0.45 ppm and 0.39-0.66 ppm in intensely cultured shrimp farms in Thailand. Tookwinas *et al.* (1994) also reported the mean NH_3-N levels of 0.19 mg/l from shrimp farms in Thailand. Prabhu *et al.* (1999) noted NH_3 concentration in the range of 0.39-1.0 ppm in shrimp culture ponds of Tamil Nadu, which were higher compared to the levels of the present study.

The NH_3-N levels were highest in semi-intensive system due to the increased input of feed and with increasing DOC. Rao (1997) recorded 0.3757-1.7143 ppm NH_3 in shrimp ponds of Karnataka which is quite higher compared to the levels recorded in West Bengal shrimp culture ponds. It is possible that the

reduced ammonia build up due to it being oxidized to NO_2 by resident NH_3 oxidizers.

Nitrite-N levels, although differed significantly ($P<0.05$) among the systems, were below the safe levels, i.e., <0.25 ppm (MPEDA, 1992) in all the systems. Their levels were found to differ significantly between the first 60 DOC and later 60 DOC probably due to increased availability of substrate and microbial activities. Nitrate-N levels, though differed significantly ($P<0.05$) among the culture systems, were less in all the systems. The values were lower compared to the levels reported by Briggs and Funge-Smith (1994) from shrimp ponds, which had mean value of 0.04-0.10 and 0.08-0.16 ppm of NO_3-N. Prabhu *et al.* (1999) observed NO_2 concentration in the range of 0.009-0.027 ppm and NO_3 concentration in the range of 0.018-0.028 ppm in shrimp culture ponds which are in close agreement with the present study. Rao (1997) noted 0.0015-0.0316 ppm NO_2-N and 0.0016-0.213 ppm ppm NO_3-N from culture ponds of Karnataka, which are comparable to the observations of the present study.

5.3. Efficacy of commercial bioremediators and products in removing total ammoniacal nitrogen (TAN) in microcosm experiments

5.3.1. Efficacy of commercial products including bioremediators

The results presented in Fig 2a, revealed that all the commercial products are capable of removal of total ammonical nitrogen (TAN) from 5 ppm level at temperature 20±5°C, although at varying degrees. Biocult was the most effective, removing 79.3% TAN in 7 days. This was followed by Gasonex, Epicin and Zeolite with a removal of 69.68%, 65% and 63.18% TAN, respectively. In control experiment the TAN removal was only 50.6%. Nevertheless, the differences in the levels of TAN were insignificant ($P>0.05$). The results revealed that the commercial products are not efficiently oxidizing NH_3-N in microcosm

environment at temperature 20±5°C as there has been a moderate or low increase in the levels of NO_2 or NO_3 (Figs. 2b and 2c). The levels of NO_2 and NO_3 also differed insignificantly ($P>0.05$) in all the experimental and control tanks (Table 8). According to Moriarty (1996) lack of right strains of bacteria or presence of non-viable bacteria in commercial products would result in unsuccessful TAN removal. The control tanks achieved a removal of 50.6% TAN in 7 days, probably due to volatilization of NH_3 or utilization by resident microflora to some extent. Since Biocult and Epicin gave comparatively better results they were selected for further experimentation.

5.3.2. Effect of initial ammonia levels on the efficacy of commercial products

The microcosm experiments were carried out at temperatures in the range of 25-35°C (30±5°C). Both Biocult and Epicin were capable of removal of TAN to the tune of 86-90% (max) at 1-3 ppm initial level of NH_3. At 4 ppm level it could remove only 60% TAN (Figs. 3a, 4a, 5a and 6a; Table 6). The observed significance of difference ($P<0.05$) in the removal of TAN at varying levels of initial NH_3 concentration, thus revealed that at initial high NH_3 levels the commercial products failed to remove majority of the TAN. This was also observed in the control tanks with varying levels of NH_3. At 1 ppm NH_3 level, nearly 50% of TAN was removed; while in others, it was in the range of 28-37% (Figs. 3a, 4a, 5a and 6a; Table 6). The removal of TAN was, however, low in control tanks as compared to the previous microcosm experiment (Section 5.3.1.) probably due to the variations in the environmental conditions. The higher rate of removal of TAN in Biocult treated tanks revealed the increased activity of ammonia oxidizers at higher temperature (30±5°C). The relatively high activity of *Nitrosomonas* sp in Biocult was further substantiated by the fact that the percentage increase in NO_2 was higher at all the four different NH_3 levels. The significant differences in TAN removal rates in Epicin treated tanks with 1-4 ppm

NH_3 levels could be attributed to the variations in the C:N ratio. The efficacy of Epicin was reported to be maximum when a C:N ratio of >10 is maintained (Briggs and Turnbull, 1995). The difference in C:N ratio in tanks with 1-4 ppm NH_3 levels was, therefore, the main cause for differences in the TAN removal rates of Epicin. The observed differences in the control tanks could be due to the varying degrees of volatilization of NH_3 or utilization by resident microflora.

The observed significant differences in NO_2 levels in treated and control tanks could be attributed to the differences in the levels of resident nitrifying bacteria that utilize NH_3 and oxidize it to NO_2 and also the role of environmental factors could not be ruled out. The results are in sharp contrast to Briggs and Turnbull (1995) who reported on the efficacy of Epicin in removing NO_2 concentration. The significant differences ($P<0.05$) in the levels of $N0_2$ in Biocult and Epicin treated tanks revealed the differences in the microbial activity. Probably, the Biocult contained high levels of ammonia oxidizers that oxidizes NH_3 effectively to produce NO_2 and Epicin contained bacteria that utilize NH_3 as a source of N_2 for growth.

The NO_3 levels increased in all the treatment tanks, but showed a general decreasing trend in control tanks (Figs. 3c, 4c, 5c and 6c) underscoring the role of microbes in the cycling processes. Further, the initial NH_3 levels had no effect on the NO_3 levels both in experimental and control tanks (Table 8).

5.4. Development of bioremedial measures using indigenous microflora

5.4.1. Efficacy of indigenous flora on TAN removal in microcosm experiments

Among the different indigenous bacterial flora tried in reducing TAN from 5 ppm level at 20±5°C, the most efficient was the mixture of *Nitrosomonas* sp and *Nitrobacter* sp, which removed about 96.5% of TAN. This was followed by

Bacillus sp and a microbial consortium (Microcon) with a removal of 95.73% and 70.84% TAN, respectively. While in control tanks only 50% of TAN was removed (Fig 7a and Table 6). The better efficiency of *Nitrosomonas* sp and *Nitrobacter* sp in combination, and *Bacillus* sp alone in removal of TAN from 5 ppm is justified by the capability of *Nitrosomonas* sp and *Bacillus* sp to oxidize NH_3 efficiently (Watson *et al.*, 1981; Koops and Muller, 1991). The capability of *Bacillus* sp to utilize NH_3 as nitrogen source for its growth has also been reported (Chandrika, 1999). The observed poor results with Microcon could be attributed to the low levels of NH_3 utilizing bacteria present and also due to the competitive inhibition by the heterotrophs present in the consortium.

The levels of NO_2 and NO_3 at 5 ppm levels of NH_3 were statistically insignificant ($P>0.05$) in both experimental and control tanks. Nevertheless, the observed high increase in the levels of NO_2 in tanks seeded with a mixture of *Nitrosomonas* sp and *Nitrobacter* sp and Microcon suggested that the NH_3 is getting oxidized to NO_2 by nitrifying bacteria (Koops and Muller, 1991).

5.4.2. Effect of initial ammonia levels on the efficacy of bioremediators with indigenous flora

The results presented in Figs. 8a, 9a, 10a and 11a and Table 6, revealed that the indigenous microflora in combination or alone were capable of removal of TAN to the tune of 85-99% within a week at 30±5°C. Significant differences ($P<0.05$) in TAN removal were observed with varying levels of initial NH_3 concentration. *Bacillus* sp was capable of removal of 88-95% of TAN within a week. The rate of removal was however, affected by the initial NH_3 levels, probably due to the varying degrees of oxidation of NH_3. Likewise, the observed significant difference in the TAN removal rates by *Nitrosomonas* sp and *Nitrobacter* sp mixture at 1 and 2 ppm levels of NH_3 compared to 3 and 4 ppm levels of NH_3 could be due to the fact that the activity of nitrifying bacteria is substrate dependent. Similar results were observed with *Nitrosomonas* sp and

Bacillus sp mixture. The results of the present study, in general, revealed that the TAN removal rate is affected by higher initial NH_3 concentration and vary with varied microbial activity. On the other hand, the initial NH_3 levels did not have any effect on the TAN removal rate (Figs. 8a, 9a, 10a and 11a; Tables 6 and 8) of *Nitrosomonas* sp, *Bacillus* sp and Microcon mixture, and was the most effective among all, removing more than 97.5% of TAN. This could be due to the synergistic effect of all the 3 groups to oxidize NH_3 and/or to utilize it as nitrogen source for growth. It has been reported that the coculture with a heterotroph is able to increase the nitrifying activity of an autotrophic *Nitrosomonas* sp strain, or to reduce its lag phase (Kuenen and Gottschall, 1982; Kaplan, 1983). The differences in the mechanism of NH_3 utilization by nitrifying bacteria and/or different rate of substrate utilization by indigenous flora (Watson *et al.,* 1981; Koops and Muller, 1991; Chandrika, 1999) could explain the observed differences in the NO_2 and NO_3 levels.

5.5. Development and evaluation of bioremediator, NB Mix, in simulated pond condition

The results presented in Figs. 12a, 12b and 12c and Table 7 revealed that none of the commercial products and the NB Mix containing indigenous bacteria was effective in reducing TAN. There existed no significant differences in NH_3 levels of both experimental and control tanks. The introduction of 4 numbers of tilapia (stocking density $100/m^3$) and daily feeding schedule in tanks resulted in an increase of NH_3 to about $\approx$5 ppm during the period of acclimation. The seeding of bioremediators in tanks containing about 5 ppm NH_3 did not show any reduction in TAN, rather the TAN levels increased in both treatment and control tanks. It is important to mention here that in microcosm experiments significant reduction was observed in TAN when the initial NH_3 was kept about above 5 ppm (Figs. 2a and 7a). The ineffectiveness of NB Mix and the commercial products on the

removal of TAN in this study could be attributed to the low level of bacterial inoculum, high stocking density of fish and excess production of NH_3 from the waste food, fish excreta and decomposition of other organic matter. Further, high initial NH_3 levels were found to influence the TAN removal rate in microcosm experiments.

The results of the present study also revealed a significant difference ($P<0.05$) in NO_2 levels and no marked differences in NO_3 levels ($P>0.05$) between the control and the treated tanks (Table 8). On the contrary, Shan and Obbard (2001) reported that the immobilized nitrifying bacterial culture was highly proficient in removing TAN when TAN was continuously supplied at concentrations similar to that produced in aquaculture as a result of shrimp excretion and food addition to pond water.

A number of techniques have been developed for the control of TAN concentration in aquaculture system in recent years. Submerged flow biofilters (Abeysinghe *et al.,* 1996), high rate linear-path trickling nitrification filters (Twarowska *et al.,* 1997), bench-scale fluidized bed bioreactors (Ng *et al.,* 1996), continuous bioreactors using immobilized alginate beads (Kim *et al.,* 2000), pellet immobilization of nitrifying bacteria (Shan and Obbard, 2001) and other TAN removal approaches/products (Porubcan, 1991a, b; Qiao Zhenguo *et al.,* 1992; Li Zhuojia *et al.,* 1997; Chandrika, 1999; Prabhu *et al.,* 1999; www.regenesis.com; www.alken-murray.com; www.supplyindia.com/microactive.htm) have all been evaluated with varying degrees of success. Achieving a high rate of TAN removal under conditions of continuous TAN production can be a major challenge. At an experimental prawn farm Chin and Ong (1997) achieved only a 25% TAN removal rate by combining a secondary treatment system with effluent biofiltration.

Commercial bioremediator products for aquaculture use are available in plenty and a few of these are AQUA.BACTA.AID.; ACCELOBAC; Epicin; Bactaclean-ALGAE, Type 2; Alken Clear-Flo®; Sanjiban Microactive; NS Series

Super SPO; Bioklean-MX 1, etc. Their efficacy and success rates are contradictory (Boyd *et al.,* 1984; Tucker and Lloyd, 1985; Ehrlich *et al.,* 1988; Qiao Zhenguo *et al.,* 1992; Anon, 1993; Anon, 1995; Funge-Smith and Hawthorn, 1996; Moriarty, 1996; Li Zhuojia *et al.,* 1997; Prabhu *et al.,* 1999; www.regenesis.com; www.alken-murray.com; www.supplyindia.com) and questionable as suppliers of such products often overrate their potential (Young, 1976). Stephenson and Stephenson (1992) opined that inadequate substrate concentration and cell density, interspecific competition with indigenous microorganisms leading to growth inhibition, and an insufficient acclimatization period to affect bioremediation may lead to failure of inocula to function in aquaculture as they do in axenic culture.

In general, the effective control of TAN concentration in shrimp pond water with indigenous bacterial flora under laboratory condition has significant and positive implication with respect to shrimp productivity and removal of TAN from shrimp pond water. The indigenous bacterial flora can induce immediate TAN removal without the constraint of acclimatization and interspecific competition associated with the use of proprietary and non-indigenous cultures (Shan and Obbard, 2001). The challenge in maintaining a viable culture of indigenous bacteria at high cell density in active growth phase is a key factor in providing an effective treatment for shrimp culture pond water. The results of the present study would provide a basis for the future research and refinement of techniques on bioremediation in shrimp aquaculture.

Chapter - 6

SUMMARY

6. SUMMARY

The present study was carried out to record the dynamics of nutrient cycling in the shrimp culture ponds of West Bengal, through quantification of bacteria involved in nitrogen and sulphur cycles. Further, attempts were made to isolate and identify bacteria, which help in bioremediation and to study the efficiency of these bacteria in causing mineralization in microcosms. The results obtained have been summarized as follows.

1) Levels of heterotrophic bacteria were higher in pond sediment than in pond water of different shrimp farming systems.
2) Bacteria involved in nitrogen cycle were present in all the culture systems and there existed significant differences in their counts among the systems.
3) The counts of bacteria involved in the sulphur cycle were considerably low and also exhibited significant differences among the systems.
4) All the physico-chemical parameters except salinity were within the safe levels for culture of shrimp in the different shrimp farming systems.
5) Nitrification bacteria and *Bacillus* sp alone or in combination were effective in bringing down the total ammoniacal nitrogen (TAN) levels in microcosm experiments.
6) Microcon, a bacterial consortium developed from the samples of shrimp farming systems, was capable of utilizing ammonia.
7) There existed significant differences in TAN removal rates between different microbial flora at different initial levels of ammonia.
8) *Bacillus* sp, *Nitrosomonas* sp and Microcon in combination was the most effective in reducing TAN levels in microcosm experiment.

9) The TAN removal efficacy of commercial products was less when compared to indigenous microbial flora.

10) The *Nitrosomonos-Bacillus* mixture (NB Mix) immobilized in sand-clay particles and the commercial bioremediators failed to reduce TAN levels in simulated pond condition, with a stocking density of 100 fishes /m^3.

Chapter - 7

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7. BIBLIOGRAPHY

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Chapter - 8

ANNEXURE

8. ANNEXURE

Annexure – 1. Description of different shrimp culture systems of West Bengal

Culture system	Source of seed	Feed used	Source of water	Management practices followed
Semi-intensive	Hatchery, rarely wild	Formulated feed C.P. Aqua feed	Sea water (Bay of Bengal)	High stocking density: >18 nos/m^2; provision of aerator; frequent water exchange; high feed input; reservoir for water treatment; water depth: 1m; regular monitoring of the system.
Modified extensive	Hatchery and wild	Formulated feed C.P. Aqua feed, Water Base	Brackish water (Rasulpur river and canal)	Stocking density: 6-18 nos/m^2; aeration by pumping; medium water exchange; high feed input; with or without reservoir; water depth: 1m; regular monitoring of the system.
Improved traditional	Hatchery and wild	Locally prepared feed	Sea water (Bay of Bengal)	Stocking density: 4-6 nos/m^2; no provision for aeration and monitoring; need based water exchange; water depth: 1m.
Stagnant pond culture	Hatchery and wild	Formulated feed C.P. Aqua feed, Avanti, Wockhardt	Brackish water (Rasulpur canal)	Stocking density: 8-12 nos/m^2; no provision for inlet and outlet and aeration; no provision for water exchange and monitoring; high feed input; water depth: 1.5-2 m; filling and draining by pumping.
Traditional	Hatchery and wild	Mostly nil; occasionally locally prepared feed	Brackish water (Bidyadhari river and tributaries)	Stocking density: $\leq$ 4 nos/m^2; no provision for aeration and monitoring; no provision for water exchange; water depth: 0.75-1 m; partial stocking and partial harvesting throughout the culture period.