Nitrifying Bioreactors Integrated into Shrimp and Prawn Hatchery Systems: Molecular Characterization of the Nitrifying Consortia, Reactor Kinetics, Modeling and Validation

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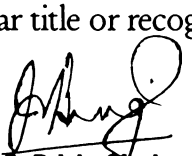
March 2008

Certificate

This is to certify that the research work presented in this thesis entitled **"Nitrifying Bioreactors Integrated into Shrimp and Prawn Hatchery Systems: Molecular Characterization of the Nitrifying Consortia, Reactor Kinetics, Modeling and Validation"** is based on the original work done by Mr. Rejish Kumar. V.J. under our guidance, at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 682016, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.



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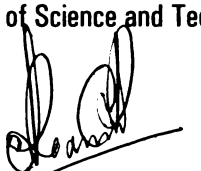

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DECLARATION

I hereby do declare that the work presented in this thesis entitled "Nitrifying Bioreactors Integrated into Shrimp and Prawn Hatchery Systems: Molecular Characterization of the Nitrifying Consortia, Reactor Kinetics, Modeling and Validation" is based on original work done by me under the guidance of Prof. I.S. Bright Singh, Coordinator, National Centre for Aquatic Animal Health, and Co–guidance of Dr. Rosamma Philip, Senior Lecturer, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Kochi 682016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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LIST OF ABBREVIATIONS

AmoA	Ammonia monooxygenase subunit A
AMONPCU-1	Ammonia Oxidizing Consortia for Non-Penaeid Culture
AMOPCU-1	Ammonia Oxidizing Consortia for Penaeid Culture
Anammox	Anaerobic Ammonium Oxidation
ANOVA	Analysis of variance
AOA	Ammonia Oxidizing Archaea
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical Oxygen Demand
CANON	Completely Autotrophic Nitrogen Removal Over Nitrite
COD	Chemical Oxygen Demand
cPCR	Competitive PCR
DAPI	4,6-diamidino-2-phenylindole
Deamox	Denitrifying Ammonium Oxidation
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
ELISA	Enzyme-Linked Immunosorbent Assay
FISH	Fluorescence In Situ Hybridization
HAO	Hydroxylamine Oxidoreductase
HRT	Hydraulic Retention Time
ICM	Intracytoplasmic membrane
IHHNV	Infectious Hypodermic and Hematopoeitic Necrosis Virus
ITS	Internal Transcribed Spacer region
MPN	Most Probable Number
NBPCU	Nitrifying Bacterial Consortia Production Unit
NIONPCU-1	Nitrite Oxidizing Consortia for Non-Penaeid Culture
NIOPCU-1	Nitrite Oxidizing Consortia for Penaeid Culture
NiR	Nitrate reductase
NO_2-N	Nitrite Nitrogen
NO_3-N	Nitrate Nitrogen
Oland	Oxygen Limited Autotrophic Nitrification and Denitrification
PBBR	Packed Bed Bioreactor
PCR	Polymerase Chain Reaction
PL	Post larvae

Ppt	Parts per thousand
RAS	Recirculating Aquaculture Systems
RBC	Rotating Biological Contactors
SBSBR	Stringed Bed Suspended Bioreactor
SAHN	Sequential Agglomerative Hierarchal Nested Cluster Method
SHARON	Single Reactor High Activity Ammonia Removal Over Nitrite
TAN	Total Ammonia Nitrogen
TANin	Total Ammonia Nitrogen into the biofilter
TEM	Transmission Electron Microscopy
TGGE	Temperature Gradient Denaturing Gel Electrophoreses
TNN	Total Nitrite Nitrogen
TSV	Taura Syndrome Virus
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VTR	Volumetric TAN Removal Rates
WSSV	White Spot Syndrome Virus
YHV	Yellow Head Virus

Chapter 1

GENERAL INTRODUCTION

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The worldwide decline of ocean fisheries stocks has provided impetus for rapid growth in fish and shellfish farming, or aquaculture. Fish produced from farming activities currently accounts for over one quarter of all fish directly consumed by humans. As the human population continues to expand beyond 6 billion, it's reliance on farmed fish production as an important source of protein will also increase. It is a fast growing food sector which now accounts for almost 50% of world's food fish production (FAO, 2006). With stagnating/declining traditional fisheries, aquaculture promises the greatest potential to meet the growing demand of aquatic food. Aquaculture not only provides a sustainable source of aquatic food, but also provides meaningful livelihood to multitudes of poor since it is almost exclusively practiced in peri-urban or rural, remote areas (FAO, 2006). Over the last two decades, aquaculture has gone through major changes, from small scale home stead-level activities to large scale commercial farming, exceeding landing from capture fisheries in many areas (National Research Council, 1992; NACA/FAO, 2001)

1.1 Shrimp/prawn hatcheries

Globally, Penaeid shrimp culture ranks sixth in terms of quantity and second in terms of value amongst all taxonomic groups of aquatic animals cultivated (FAO, 2006). The economic profitability of shrimp culture leads many aquarists to risk a substantial investment in it. Breakthroughs in shrimp larviculture during the 1970s ensured abundant and low cost seed enabling intensification of shrimp culture (Fast and Menasveta, 2000).In places were warm-water aquaculture was possible the trade grew faster due to seed availability and most importantly due to the high prices the produce fetched (Pechmanee, 1997). The abundant seed availability of *Penaeus monodon* was realized through intensive larviculture in hatcheries. Intensive culture, apart from other problems, results in enhanced susceptibility of the cultured species to diseases which infact has become the biggest constraint in shrimp aquaculture (FAO, 2003). Production of the *P.monodon and P. vannamei* relies

almost exclusively on hatchery produced seed and loss due to diseases has significant impact on not only the profitability of the hatcheries, but also on grow-outs by way of increased input costs (FAO, 2003).

Freshwater prawn (*Macrobrachium rosenbergii* (De Man) or scampi) used to be very common in the natural waters. As a result of overfishing and deterioration of its habitat and spawning grounds, the natural catch has been reduced drastically and the prawn has become a luxury food item; production no longer meets consumer demand. Along this *Macrobrachium rosenbergii* is of commercial importance owing to its fast growth in subtropical and tropical regions (New, 1995). Since it has been possible to produce postlarvae in hatcheries (Fujimura and Okamoto, 1972; Ling, 1977), the prospects of its culture and consequently the number of active prawn farms have significantly increased. Moreover, there is great scarcity of freshwater prawn seed and it is a well accepted fact that rapid development of scientific prawn farming is just impossible without meeting the demand for good quality seeds. This necessitated the establishment of freshwater prawn hatcheries.

1.2 Indian scenario

The number of shrimp hatcheries in India has increased rapidly since the late 1980s. There are now approximately 300 hatcheries, mostly in the state of Andhra Pradesh State, with an average production capacity of 33 million postlarvae (PL) per year (Table.1). The total production of PL in India has increased with this hatchery development to approximately 10 billion per year in 2002-2003, requiring up to an estimated 200,000 broodstock per year (Fig.2) (FAO, 2007). In India *M. rosenbergii* is cultured in an area of 34,630 ha with an average production per ha ranging from 880 to 1250 kg. To support the industry there are 71 hatcheries spread across the country supplying 183 billion scampi seed (Bojan, 2007). A vast majority of the culture systems in India are of the extensive and traditional type, followed by semi intensive variety, and hatchery produced seed is the main source of fry for stocking the ponds (Hein, 2002).

Table.1. Number and production capacity of shrimp hatcheries in India by state (FAO, 2007)

State	*Penaeus monodon*		*Macrobrachium sp.*		Total	
	Number	Capacity (x 10^6)	Number	Capacity (x 10^6)	Number	Capacity (x 10^6)
Andhra Pradesh	148	7 882	43	1 453	191	9 335
West Bengal	2	100	9	66	11	166
Orissa	13	455	2	20	15	475
Kerala	22	484	7	53	29	537
Tamil Nadu and Pondicherry	73	2 863	8	215	81	3 078
Karnataka	13	301	0	0	13	301
Gujarat	2	45	0	0	2	45
Goa	1	20	0	0	1	20
Maharashtra	6	325	2	20	8	345
Total	280	12 475	71		351	14 302

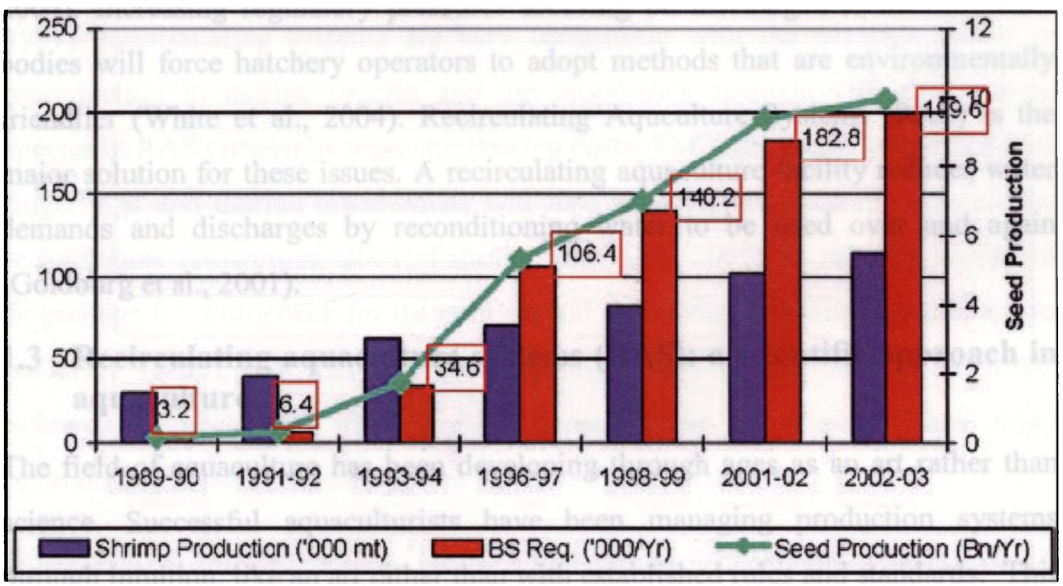

Fig.1. Shrimp production, seed production and brood stock requirements for India (FAO, 2007)

However, the sustainability of the sector is still hampered by many problems, foremost among these being a reliance on wild-caught broodstock whose supply is limited both in quantity and in seasonal availability and that are often infected with pathogens. The current low quality of hatchery produced PL, due to infection with white spot syndrome virus (WSSV) and other pathogens entering the hatcheries via infected broodstock, contaminated intake water or other sources due to poor hatchery management practices, including inadequate biosecurity, is a major obstacle to achieve sustainable shrimp aquaculture in India and the Asia-Pacific region (FAO, 2007)

The environmental implications of growth in aquaculture production are being increasingly getting realized. Some types of aquaculture activity, including shrimp and salmon farming have shown to cause potential damage to ocean and coastal resources through habitat destruction, waste disposal, exotic species introduction and pathogen invasions and may further deplete wild fisheries stocks (Naylor et al., 2000). Thus in recent years, there has been growing concern over the impacts of aquaculture operations (Harache, 2002; Cranfold et al., 2003; Johnson et al., 2004). Increasing regulatory pressures focusing on discharges to natural water bodies will force hatchery operators to adopt methods that are environmentally friendlier (White et al., 2004). Recirculating Aquculture Systems (RAS) is the major solution for these issues. A recirculating aquaculture facility reduces water demands and discharges by reconditioning water to be used over and again (Goldburg et al., 2001).

1.3 Recirculating aquaculture systems (RAS): a scientific approach in aquaculture

The field of aquaculture has been developing through ages as an art rather than science. Successful aquaculturists have been managing production systems through intuition, like an art rather than with established rules and standards. This has acted in fact as barriers to the introduction of modern technologies and management practices used in similar industries (Lee et al., 2000). It has been realized that aquaculture is a science, the physiology and behavior of the cultured

species can be described and manipulated using scientific and engineering methods. This particularly is true for recirculating aquaculture systems that are comparable to simple mesocosms, making it possible to quantify accurately environmental conditions and their effects on physiological rates.

Recirculating systems have been identified as one of the main areas for research in aquaculture (NOAA, 2001; Martin, 2002). A recirculating aquaculture facility reduces water demands and discharges by reconditioning water to be used over and again (Goldburg et al., 2001). Better food conversions are achievable with a recirculating aquaculture system (RAS) suggesting less waste generation from feed (Lossordo et al., 1998). RAS can reduce the effluent waste stream by a factor of 500-1000 (Chen et al., 1997; Timmons et al., 2001) allowing existing operations to upgrade and expand and comply with future regulations.

The applicability of RAS technologies to production of marine species has been amply demonstrated (Manthe et al., 1988; Davis and Arnold, 1998). In the saltwater systems, RAS plays an important role in the production of healthy, properly sized fingerlings for stocking out in net pens or ponds (Fielder and Allan, 1997). Recirculating systems are very compatible with the complex nature of reproduction in marine species and the broodstock fecundity of most marine species in RAS outweighs waste processing costs. The high values associated with fingerlings and marine ornamentals will also promote adoption of recirculating technologies (Howerton, 2001; Palmtag and Holt, 2001) as reliable supply of fingerlings is a bottleneck for the commercial production of marine species as sea bass, sea bream, flat fish and cobia among others (Wantanabe et al., 1998; Schwarz et al., 2004). The higher market prices of marine fishes make recirculating systems a better option for sustained production.

Biosecurity issues are another important matter for consideration in the use of RAS by the hatchery operators (Otoshi et al., 2003; Pruder, 2004). Water recirculation dramatically reduces the possibility of pathogen introduction (Davis, 1990, Goldburg et al., 2001). As an example, high biosecurity is critical to prevent

introduction of diseases that have plagued the shrimp industry in recent years such as the WSSV, YHV, IHHNV and TSV, the current devastating problems in shrimp culture. A number of factors pressurize the hatcheries to be away from coasts. In such situations disposal of salty wastes after becomes a major environmental issue and establishment of recirculation systems with extended reuse of water is the only option. Two fundamental obstacles have prevented the full potential of recirculating technologies from being realized. First, cost effective design and management strategies that minimize complexity and reduce the energy and labour intensity (Turk and Lee, 1991; Westerman et al., 1993; Lee, 1995; Malone and De Los Reyes, 1997) and second , management strategies that did not encourage the adoption of new technology by the aquaculture industry.

The most prominent characteristic of any RAS is the efficient biofilter which will maintain water quality preventing accumulation of toxic metabolites, the most notorious of which are ammonia and nitrite. High levels of ammonia and nitrite undermine commercial production objectives as the toxic impacts are manifested through impaired growth or chronic diseases (Manthe et al., 1985; Cheng et al., 2004; Svoboda et al., 2005). However, nitrate is relatively harmless to the aquatic organisms (Tomasso, 1994).

1.4 Ammonia and nitrite toxicities in shrimp/prawns

1.4.1 Ammonia

Total ammonia nitrogen (TAN) is the key limiting water quality parameter (Losordo and Westers, 1994; Lyssenko and Wheaton, 2006; Fontenot et al., 2007). Ammonia and its intermediate product of oxidation, nitrite, are the most common toxicants in culture systems and are toxic to fish, molluscs and crustaceans (Colt and Armstrong, 1981). Ammonia is very toxic to penaeid prawns (Chin and Chen 1987) and can build up to a critically high concentration during intensive prawn culture (Chen et al., 1988). The toxic effects of ammonia have been demonstrated for several cultured crustaceans (Kir et al., 2004; Koo et al., 2005) and found more pronounced in early developmental stages. In young shrimp larvae, concentrations of non-ionic ammonia as low as 0.01mg/L can

result in mortalities besides causing pathological disturbances as well as depressing growth rates (Hamid et al., 1994; Ostrensky and Wasielesky Jr., 1995; Liu and Chen, 2004). It has been found that ammonia and nitrite increase exponentially both in the hatchery and in the grow-out farm, even with frequent water replacement (Chen *et al.*, 1986, 1989). In a culture system, ammonia appears in water from two sources, the ammonification of un-consumed foods by heterotrophic bacteria, and transamination and deamination of catabolic products of organic nitrogen ingested and assimilated by cultured animals (Armstrong, 1978). Heterotrophic oxidation of organic material can result in elevated ammonia concentrations (Boyd, 1990). In an aqueous ammonia solution, unionized ammonia (NH_3) exists in equilibrium with ionized ammonia (NH_4^+) and hydroxide ions. The un-ionized form is usually toxic, as it has high lipid solubility and is able to diffuse quite readily across cell membranes (Armstrong et al., 1978; Thurston et al., 1981) and the proportion of which are pH –dependent (Trussel, 1972). The proportion of NH_3 to NH_4^+ in water increases with increase in water temperature and pH, and with decrease in salinity (Trussell, 1972). Chen et al., (1986) observed that ammonia can increase to more than 0.8 mg/L ammonia-N (0.079 mg/L NH_3-N) during the development of larval prawns in a hatchery, even with frequent water replacement.

Toxicities of ammonia and nitrite to *P. monodon* larvae were reported (Chin and Chen, 1987; Chen and Chin 1988). Chin (1992) emphasized the adverse effects of ammonia on oxygen supply to prawn tissues. Accumulation of ammonia in water may retard shrimp growth and in extreme cases cause death (Wickins, 1976; Armstrong et al., 1978; Chen et al., 1990). The unionized ammonia affects growth, central nervous system function, ionic balance, energy metabolism and survival (Heath, 1995; Wicks et al., 2002; Foss et al., 2003). Elevated environmental ammonia-N has been reported to affect growth and moulting (Chen and Kou, 1992), oxygen consumption, ammonia-N excretion (Chen and Lin, 1992), and Na^+, K^+-ATPase activities of penaeids (Chen and Nan, 1992). Ammonia has also been reported to affect the immune responses of *Litopenaeus*

stylirosytris (Le Moullac and Haffiner, 2000) and *M.rosenbergii* (Cheng et al., 2002). Ammonia and nitrite toxicity in *Macrobrachium rosenbergii* adults and larvae have been investigated by various researchers (Chen and Lee, 1997; Cavalli et al., 2000; Wang et al., 2004; Naqvi et al., 2007) and felt the need for their regulation for successful larval production. However, nitrate is relatively harmless to the cultured aquatic organisms (Tomasso, 1994) and it has not been a limiting factor for *Macrobrachium* larviculture (Mallasen et al., 2004). Ammonia in water caused a depression in the immune response and an increase in mortality of *Litopenaeus vannamei* from the *Vibrio alginolyticus* infection (Liu and Chen, 2004). Phagocytic activity and clearance efficiency decreased when *M.rosenbergii* was exposed to ammonia-N at 0.55mg/L or higher, supports the view that the susceptibility of this species to *Lactococcus garvieae* infection is enhanced by suppression of the prawns' immune system in these concentrations of ammonia (Cheng et al., 2003).

Studies on haemolymph ammonia concentrations by Chen and Kou (1992) stated that, the concentrations of haemolymph ammonia can be used as an index of ammonia loading for *P. monodon* in intensive culture system. The tolerance of larval *P. monodon* to ammonia increased as the larvae metamorphosed from the nauplius to the postlarval stage. A more conservative estimate of the "safe level" for rearing larval *P.monodon* was calculated on the basis of an estimated 96-h LC_{50} for the nauplius to be 0.13 mg/L ammonia-N (0.01mg/L NH_3-N) and there was no direct relationship between shrimp development and its tolerance to ammonia and that eggs, zoeae and post larvae are very sensitive to ammonia (Chin and Chen, 1987). The "safe level" for rearing *P. paulensis* was estimated to be 0.03 mg/L NH_3-N. In general, *P. paulensis* is less resistant to ammonia than other species of shrimp (Antonio Ostrensky and Wilson Wasielesky Jr., 1995). Differences in ammonia tolerance were observed in early larval stages and remained evident throughout larval development. Based on this, a short-term ammonia toxicity test developed by Cavalli et al. (2000) proved to be a valuable, sensitive and reproducible criterion for the establishment of larval quality.

1.4.2 Nitrite

Nitrite, an intermediate product and important component in the nitrogen cycle, may build up to an unusually high concentration (0.33mM) in pond water (Chen et al., 1989), or in recirculated water due to malfunction of biofilters (Liao and Mayo, 1974; Eddy and Williams, 1987). Elevated concentration of nitrite may occur in water receiving nitrogen wastes from sewage plants and incomplete reaction in bacterial nitrification and denitrification process. Nitrite exists in aqueous solution in two forms: the un-ionized nitrous acid (HNO_2) and the ionized nitrite (NO_2^-). As with ammonia the equilibrium between nitrite and nitrous acid is primarily determined by environmental pH. Like ammonia, the un-ionized form of the toxin is freely diffusible across gill membranes, while the ionized form is not. Very little of the nitrous acids form is present at pH values commonly seen in aquaculture systems. The vast majority, in excess of 99.9% of the total nitrite, is in the non diffusible ionized form (Chen et al., 1989).

Elevated nitrite can retard growth, increase the rate of moulting and, in extreme cases cause death of decapod crustaceans (Armstrong et al., 1976; Chen and Lin , 1991; Chen and Chen, 1992). Nitrite is harmful to larvae as it causes reduction of hemolymph oxyhemocyanin (in *Penaeus monodon*) with concomitant increase in the partial pressure of oxygen (pO2) in haemolymph and reduced oxygen affinity (p50) (Cheng and Chen, 1995). However, it is less toxic than ammonia (Alcaraz et al., 1999) and, only under conditions of long term exposure the toxicity is found manifested (Wheaton et al., 1991) in the reared animals. Working on combined species of penaeid shrimps (*Penaeus aztecus, P. japonicus, P. occidentalis, P. orientalis, P. schmitti* and *P.setiferus)*, Wickins (1976) reported that the 48-hr LC_{50} value was 12.1 mM and 243 mM for nitrite and nitrate respectively. *M.rosenbergii* in a 24-hr exposure to 0.71mM nitrite, showed an accumulation of hemolymph nitrite to 2.16 mM (Chen and Lee, 1997). The ability to concentrate nitrite in the blood or hemolymph varies widely among species and correlates with the susceptibility of species to nitrite-induced mortality and methemoglobinemia.

Nitrite toxicity is ameliorated mainly by increased concentrations of external Cl^- in most teleost fishes. The 96 h LC_{50} value of nitrite on 10-14 day-old larvae of *M.rosenbergii* at 12 ppt was found to be 8.6 mg nitrite-N per liter (Armstrong et al., 1976). However little information is available on the nitrite toxicity to prawns at different salinities or external Cl^- concentrations (Chen and Lin, 1991).

Considering the above described toxicities of ammonia and nitrite, the most important segment of any RAS should be an efficient biofilter capable for the removal of these toxic metabolites from the system through the major biological process; nitrification.

1.5 Biological nitrogen removal

Since wastewater discharges containing nitrogen can be toxic to aquatic life, causing oxygen depletion and eutrophication in receiving waters, and affect chlorine disinfection efficiency, reduction of nitrogen levels from the discharge becomes necessary (Metcalf and Eddy, 2003). Nitrogen compounds can be removed from wastewater by a variety of physicochemical and biological processes. As biological nitrogen removal is more effective and relatively inexpensive, it has been widely adopted in favour of the physicochemical processes (USEPA, 1993). The conventional biological nitrogen removal (nitrification and denitrification) proceeds slowly due to low microbial activity and yield. The process is generally performed on wastewater containing low nitrogen concentration. In the past few years, several novel and cost-effective biological nitrogen removal processes have been developed, including partial nitritation, nitrifier denitrification, anaerobic ammonium oxidation (the Anammox process), and its combined system (completely autotrophic nitrogen removal over nitrite, CANON) (Jetten et al., 2002).

1.5.1 Conventional nitrification/denitrification

In conventional treatments, the biological elimination of nitrogen from wastewater requires a two-step process, involving nitrification followed by denitrification. Nitrification implies a chemolithoautotrophic oxidation of ammonia to nitrate

under strict aerobic conditions and is conducted in two sequential oxidative stages: ammonia to nitrite (ammonia oxidation) and nitrite to nitrate (nitrite oxidation). Each stage is performed by different bacterial genera which use ammonia or nitrite as the energy source and molecular oxygen as the electron acceptor, while carbon dioxide is used as the carbon source. As the second step, denitrification is generally performed by a heterotrophic bioconversion process under anaerobic (anoxic, precisely) conditions. The oxidized nitrogen compounds (NO_2^- and NO_3^-) are reduced to gaseous dinitrogen by heterotrophic microorganisms that use nitrite and/or nitrate instead of oxygen as electron acceptors and organic matter as carbon and energy source.

1.5.1.1 Chemolithoautotrophic nitrifiers

Nitrification protocols for the manufacture of nitrates by composting organic matter in soil has existed for many centuries: from the tenth century in China and from the twelfth century in Europe (Macdonald, 1986). However, the process was considered to be of chemical and not biological nature until the late nineteenth century when Pasteur suggested that nitrification was of biological origin (Pasteur, 1862). During 1891 Winogradsky succeeded in isolating a nitrite-oxidizing bacterium putting end to the debate whether nitrification was a one-stage process (carried by a single bacterium) or a two-stage process (carried by two distinct classes of microorganisms) (Macdonald, 1986).

Nitrification, the biological oxidation of reduced forms of inorganic nitrogen to nitrite and nitrate, is catalyzed by two physiological groups of bacteria. Ammonia-oxidizing bacteria, which use ammonia and not ammonium as substrate (Suzuki et al., 1974), gain energy from oxidation of ammonia to nitrite, and nitrite-oxidizing bacteria thrive by oxidizing nitrite to nitrate. In sea and freshwater as well as in soil, nitrite produced by the ammonia oxidizers is immediately consumed by nitrite oxidizers and thus the nitrite concentration is extremely low in these environments (El-Demerdash and Ottow, 1983; Schmidt, 1982). Nitrate can be assimilated by plants and microorganisms. Under anoxic or oxygen-limited

conditions, nitrate is used as electron acceptor for anaerobic respiration (if organic matter is available) and thereby converted to ammonia (respiratory ammonification) or dinitrogen (denitrification).

Lithotrophic nitrifiers are Gram-negative bacteria and conventionally have been placed in the family Nitrobacteriaceae (Buchanan, 1917; Watson, 1971; Watson et al., 1989). However, phylogenetically the lithoautotrophic ammonia oxidizers, characterized by the prefix *Nitroso-*, and nitrite oxidizers, characterized by the prefix *Nitro-*, are not closely related (Teske et al., 1996; Purkhold et al., 2000). The assignment of ammonia- and nitrite-oxidizing bacteria into genera was dependent primarily upon their morphological features like cell size, shape, and the arrangement of the intracytoplasmic membranes (Watson et al., 1989). The physiological and morphological grouping of the nitrifying bacteria is in contradiction to data obtained from molecular phylogenetic studies which show at least subdivision level diversity within and between the ammonia- and nitrite oxidizers (Head et al., 1993; Orso et al., 1994; Teske et al., 1994; Ehrich et al., 1995; Purkhold et al., 2000). Comparative 16S rRNA gene sequence analysis demonstrated that all recognized ammonia oxidizers are either members of the β- or γ-subclass of Proteobacteria (Fig. 2). The genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a closely related monophyletic assemblage within the β-subclass of Proteobacteria (Woese et al., 1984; Head et al., 1993; Teske et al., 1994; Utåker et al., 1995; Pommerening-Röser et al., 1996; Purkhold et al., 2000), whereas the genus *Nitrosococcus* constitutes a separate branch within the γ-subclass of Proteobacteria (Woese et al., 1985; Purkhold et al., 2000). Among the nitrite oxidizers, the genera *Nitrobacter*, *Nitrococcus* and *Nitrospina* were assigned to the α-, γ, and δ-subclass of Proteobacteria, respectively (Orso et al., 1994; Teske et al., 1994). Nitrite oxidizers of the genus *Nitrospira* are affiliated with *Nitrospira* -phylum, which represents an independent line of descent within the domain Bacteria (Ehrich et al., 1995).

Significant differences between ammonia- and nitrite-oxidizing bacteria are also indicated by the fact that both physiological groups possess very different key enzyme systems for the energy-gaining oxidation of ammonia and nitrite. With the exception of the nitrite oxidizers of the genera *Nitrospina* and *Nitrospira*, all known nitrifiers are closely related to phototrophs and thus presumably originated in several independent events by conversion of photosynthetic ancestors to chemolithotrophs (Teske et al., 1994).

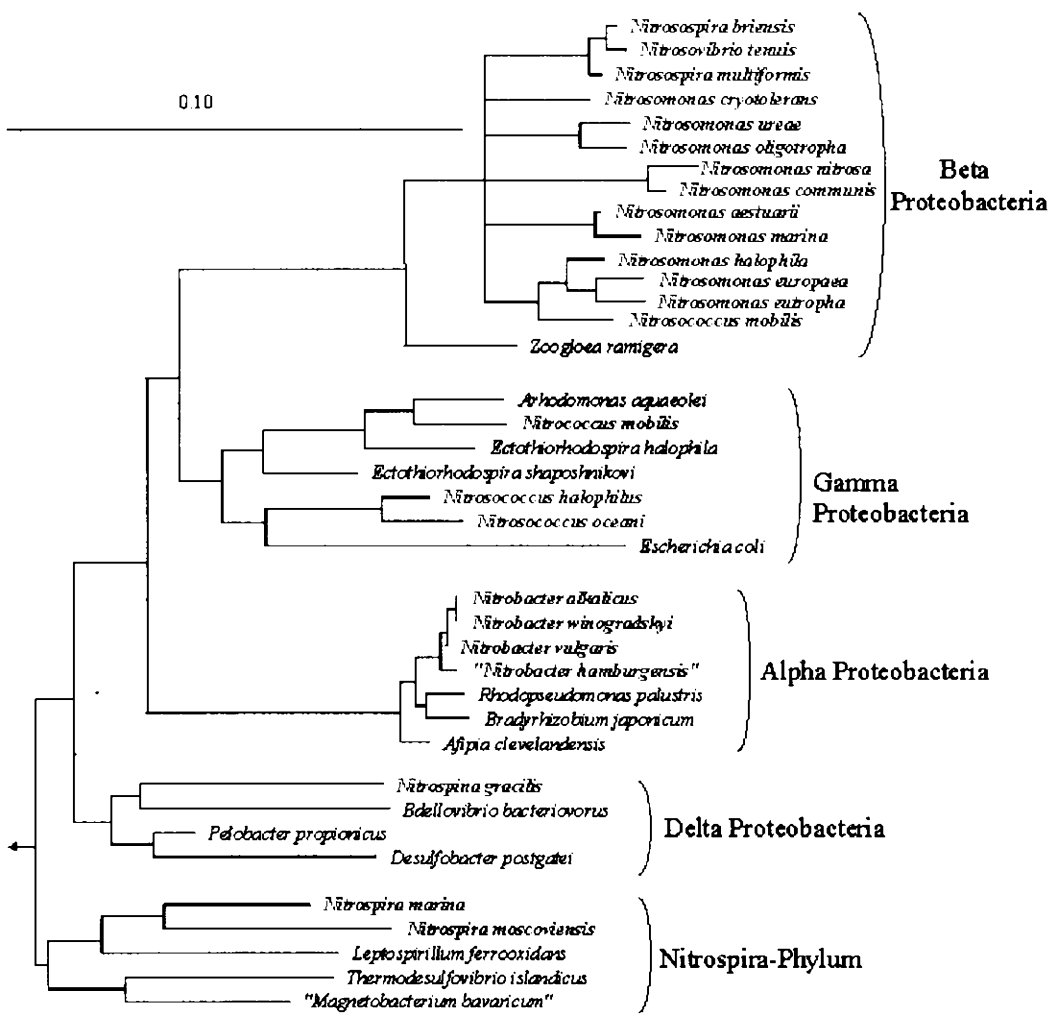

Fig. 2. 16S rRNA-based tree reflecting the phylogenetic relationship of ammonia- and nitrite-oxidizing bacteria. Ammonia oxidizers are labeled green and nitrite oxidizers are depicted in red (Bock and Wagner, 2006)

Nitrifying bacteria are present in oxic and even anoxic environments. They are widely distributed in fresh water, seawater, soils, on/in rocks, in masonry, and in wastewater treatment systems. Nitrifiers also could be enriched or isolated from extreme habitats like heating systems with temperatures of up to 47°C (Ehrich et al., 1995) and permafrost soils up to a depth of 60 m at a temperature of down to -12°C. Although the pH optimum for cell growth is 7.6–7.8, nitrifiers were frequently detected in environments with suboptimal pH (e.g., acid tea soils and forest soils at pH values below 4, but also in highly alkaliphilic soda lakes at a pH of 9.7–10.5 (Sorokin et al., 2001). Growth under suboptimal acidic conditions might be possible by ureolytic activity, by aggregate formation (De Boer et al., 1991), or as biofilms (e.g., on clay particles; Allison and Prosser, 1993). In many environments, nitrifier sensitivity to sunlight is of ecological importance. The light sensitivity of ammonia- and nitrite oxidizers increases from blue light to long wave UV (Hooper and Terry, 1974; Shears and Wood, 1985). Based on spectroscopic similarities, Shears and Wood (1985) postulated a model of the ammonia monooxygenase light inhibition similar to the three-stage catalytic cycle of the tyrosinase reaction. In *Nitrobacter*, which is more sensitive to visible light than *Nitrosomonas* (Bock, 1965), the photooxidation of *c*-type cytochromes is assumed to cause light-induced cell death (Bock, 1970).

Although *Nitrosomonas euopaea* and *Nitrobacter* sp. are the most commonly investigated ammonia- and nitrite oxidizers in laboratory studies, molecular analysis revealed that other nitrifiers are of higher importance in many natural and engineered systems. For example, stone material of historical buildings and many soil systems seem to be dominated by members of the genera *Nitrosovibrio* and *Nitrosospira*, respectively (Meincke et al., 1989; Spieck et al., 1992; Hiorns et al., 1995; Stephen et al., 1996), whereas different *Nitrosomonas* species and *Nitrosococcus mobilis* are the most abundant ammonia oxidizers in wastewater treatment plants (Juretschko et al., 1998; Purkhold et al., 2000). Interestingly, not yet cultured members of the genus *Nitrospira* and not *Nitrobacter* are the most

abundant nitrite oxidizers in sewage treatment plants and aquaria filters (Wagner et al., 1996; Burrell et al., 1998; Juretschko et al., 1998; Daims et al., 2000).

1.5.1.2 Proteobacterial ammonia oxidizers

Chemolithotrophic ammonia oxidizers were isolated for the first time at the end of the nineteenth century (Winogradsky, 1892). Since then, 16 species of ammonia oxidizers have been described (Jones et al., 1988; Koops et al., 1976; Koops et al., 1990; Koops et al., 1991; Watson, 1965), and according to DNA-DNA hybridization experiments, at least 15 additional genospecies are "hidden" in existing culture collections (Koops et al., 1991; Koops and Harms, 1985; Stehr et al., 1995). Our current perception of evolutionary relationships of ammonia-oxidizing bacteria is mainly based on comparative sequence analysis of their genes encoding the 16S rRNA and the active site polypeptide of the ammonia monooxygenase (AmoA). During the last decade, the genes for both biopolymers were sequenced for all recognized ammonia oxidizer species (Alzerreca et al., 1999; Head et al., 1993; Pommerening-Röser et al., 1996; Teske et al., 1994; Purkhold et al., 2000; Rotthauwe et al., 1995; Rotthauwe et al., 1997; McTavish et al., 1993; Horz et al., 2000) and the deduced phylogeny now provides an encompassing and relatively robust framework for assignment of 16S rDNA and *amoA* sequences of 1) ammonia oxidizer isolates (Stehr et al., 1995; Suwa et al., 1997; Utåker et al., 1995; Juretschko et al., 1998) and 2) cloned sequence fragments directly retrieved from the environment (Stephen et al., 1996; Rotthauwe et al., 1995; Purkhold et al., 2000).

According to comparative 16S rRNA sequence analysis, all recognized ammonia oxidizers are members of two monophyletic lineages within the β- and γ-subclass of Proteobacteria. The marine species *Nitrosococcus halophilus* and *Nitrosococcus oceani*, which are distantly related to methane-oxidizing bacteria, cluster together in the γ-subclass of Proteobacteria. All other ammonia oxidizers form a monophyletic assemblage within the β-subclass of Proteobacteria, most closely related to the iron-oxidizer *Gallionella ferruginea*. This lineage

encompasses the genera *Nitrosomonas* (including *Nitrosococcus mobilis*, which is actually a member of the genus *Nitrosomonas*), *Nitrosovibrio*, *Nitrosolobus* and *Nitrosospira*. It has been suggested (Head et al., 1993) and subsequently questioned (Teske et al., 1994) that the latter three genera should be reclassified into the single genus *Nitrosospira*. The nitrosomonads can be further subdivided into the *N. europaea/Nc. mobilis* cluster, the *N. marina* cluster, the *N. oligotropha* cluster, and the *N. communis* cluster (Purkhold et al., 2000). *Nitrosomonas cryotolerans* forms a separate lineage within the β-Proteobacteria. The genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* are closely related and form a cluster to the exclusion of the nitrosomonads. Similar but not identical evolutionary relationships were obtained if comparative analysis of AmoA sequences were performed (Purkhold et al., 2000).

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + H_2O + 2H^+ + 240\ kJ$$

Ammonia oxidizers are lithoautotrophic organisms using carbon dioxide as the main carbon source (Bock et al., 1991). Their only way to gain energy is the oxidation of ammonia to nitrite (Hooper, 1969). Investigations of the K_m values and pH optima indicate that ammonia (NH_3) rather than ammonium (NH_4^+) is the substrate of ammonia oxidizers (Suzuki et al., 1974; Drozd, 1976). This is in accordance with results showing that the ammonia-oxidizing enzyme might be located in the cytoplasmic membrane (Suzuki and Kwok, 1981; Tsang and Suzuki, 1982) since membranes are highly permeable to ammonia but not to ammonium (Kleiner, 1985). First, ammonia is oxidized to hydroxylamine (Kluyver and Donker, 1926) by the ammonia monooxygenase (AMO; Hollocher et al., 1981). This enzyme does not possess high substrate specificity and also oxidizes several apolar compounds such as methane, carbon monoxide or some aliphatic and aromatic hydrocarbons (Hooper et al., 1997). These compounds can act as competitive inhibitors of ammonia oxidation (Hyman et al., 1988; Keener and Arp, 1993). The second step is performed by the hydroxylamine oxidoreductase (HAO). This enzyme oxidizes hydroxylamine to nitrite (Wood, 1986). Two of the four electrons released (Andersson and Hooper, 1983) are

required for the AMO-reaction (Tsang and Suzuki, 1982), whereas the remaining ones are used for the generation of proton motive force (Hollocher et al., 1982) to regenerate ATP and NADH (Wheelis, 1984; Wood, 1986).

Most information about the reactions catalyzed by AMO originates from studies with intact cells. In addition to oxidizing ammonia, AMO can hydroxylate non-growth-supporting substrates such as hydrocarbons and alcohols (Hooper and Terry, 1973; Tsang and Suzuki, 1982; Hyman and Wood, 1983; Voysey and Wood, 1987). This is not only of theoretical interest but also could be of importance for microbial ecology (Hall, 1986). For example, pure cultures of ammonia oxidizers are able to oxidize methane, but could not grow on this alternative electron donor (O'Neil and Wilkinson, 1977; Hyman and Wood, 1983; Jones and Morita, 1983). Recent data, however, suggest that at least in the rice rhizosphere, ammonia oxidizers do not significantly contribute to the methane oxidation (Bodelier and Frenzel, 1999; Co-oxidation and Inhibition of AMO). This capability reflects structural and functional homologies between the ammonia- and the methane monooxygenase of ammonia oxidizers and methanotrophs, respectively (Bedard and Knowles, 1989).

The key enzyme of hydroxylamine oxidation, HAO (Hydroxylamine Oxidoreductase), is a multiheme enzyme, located in the periplasmic space (Olson and Hooper, 1983; Hooper et al., 1984; Hooper and DiSpirito, 1985). The enzyme complex has a relative molecular weight of 180, 315-190 and 315 and consists of an α3 oligomer closely associated with three heme centers including seven *c*-type hemes and a novel heme, P-460, per monomer (Arciero and Hooper, 1993; Bergmann and Hooper, 1994; Igarashi et al., 1997). The P-460 was found to be a CO-binding heme (Lipscomb et al., 1982). According to spectroscopic and chemical investigations, the P-460 iron resides in a heme-like macrocycle, but the presumed porphyrin must have some unusual features (Andersson et al., 1984). In total, HAO constitutes about 40% of the *c*-type heme of *Nitrosomonas europaea* (Hooper et al., 1978). The *c*-type hemes of HAO can be placed into two classes

with different oxidation-reduction midpoint potentials and protein environments, respectively (Hooper, 1984; Arciero et al., 1991; Collins et al., 1993). Ammonia-oxidizing bacteria not only catalyze aerobic ammonia oxidation but also show denitrifying activity with nitrite as electron acceptor. For example, small amounts of nitric oxide and nitrous oxide are produced during denitrification with ammonia as electron donor at reduced oxygen concentrations (Remde and Conrad, 1990; Stüven et al., 1992)

1.5.1.3 Nitrite oxidizers

Nitrite-oxidizing bacteria carry the second stage of the nitrification process, that of oxidation of nitrite to nitrate

$$NO_2^- + ½\ O_2 \rightarrow NO_3^- + 65kJ$$

All isolated chemolithoautotrophic nitrite-oxidizing bacteria (NOB) belong to one of the following four genera: *Nitrobacter* (α subclass of Proteobacteria), *Nitrococcus* (γ subclass of Proteobacteria), *Nitrospina* (δ subclass of Proteobacteria) and *Nitrospira* (distinct phylum) (Bock and Koops, 1992). The genus *Nitrobacter* was previously thought to be the main nitrite oxidizer (Bock and Koops, 1992). However, a variety of recently developed techniques made it possible to explore the composition of the nitrite-oxidizing community in the environment and to enhance our knowledge of its functioning (Wagner et al., 1996; Bartosch et al., 1999; Daims et al., 2000).

The genus *Nitrobacter* contains the four closely related species (*N. hamburgensis*, *N. vulgaris*, *N. winogradskyi* and *N. alkalicus*) within the α-subclass of Proteobacteria. Nitrite oxidizers of the genus *Nitrobacter* are phylogenetically related to *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, *Afipia clevelandensis* and the phototroph *Rhodobacter palustris* (Orso et al., 1994; Teske et al., 1994) with which *Nitobacter* shares a nearly identical arrangement of ICMs. The genus *Nitrococcus* represented by the single marine species *Nitrococcus mobilis* is, like the marine ammonia oxidizer s of the genus *Nitrosococcus*, a member of the ectothiorhodospira branch of the γ-subclass of

Proteobacteria, consistent with an assumed photosynthetic ancestry of these nitrifiers. *Nitrococcus* and *Nitrosococcus* are the only nitrite- and ammonia oxidizer s that are relatively closely related, but the closest relatives of *Nitrococcus mobilis* are the phototrophic bacteria *Arhodomonas aquaeoli*, *Ectthiorhodospira halochloris* and *Ectthiorhodospira halophila* (Teske et al., 1994). The genus *Nitrospina* with the marine *Nitrospina gracilis* as the only species (represented by two isolates, one from the Atlantic and the other from the Pacific) has been provisionally assigned to the δ-subclass of Proteobacteria and is the only member of a deep branch within this subclass (Teske et al., 1994). *Nitrospira gracilis* shows no ICMs.

The genus *Nitrospira* encompasses the marine species *Nitrospira marina* and *Nitrospira moscoviensis*, isolated from a municipal water heating system. The genus *Nitrospira* forms a monophyletic grouping with the genera *Thermodesulfovibrio*, *Leptospirillum* and with "*Magnetobacterium bavaricum.*" This phylogenetic assemblage has recently been identified as a novel phylum within the domain Bacteria and was named "*Nitrospira phylum*" (Ehrich et al., 1995). There is accumulating molecular evidence that *Nitrospira* -related nitrite oxidizers are of major importance for nitrite-oxidation in wastewater treatment plants and aquarium filters (Burrell et al., 1998; Juretschko et al., 1998; Hovanec et al., 1998; Daims et al., 2000), and also occur in many natural environments including the rhizosphere. Like *Nitrospina gracilis*, members of the genus *Nitrospira* do not possess ICMs and are apparently not closely related to phototrophic bacteria.

The key enzyme of nitrite-oxidizing bacteria is the membrane-bound nitrite oxidoreductase (Tanaka et al., 1983), which oxidizes nitrite with water as the source of oxygen to form nitrate. The electrons released from this reaction are transferred via *a*- and *c*-type cytochromes to a cytochrome oxidase of the aa_3-type. However, the mechanism of energy conservation in nitrite oxidizers is still unclear. The first product of energy conservation was shown to be NADH and not

ATP (Sundermeyer and Bock, 1981). Except for *Nitrobacter*, all other isolated nitrite oxidizers are obligate lithotrophs with nitrite serving as the only energy source. Although many strains of *Nitrobacter* are able to grow heterotrophically, growth is very inefficient and slow (Smith and Hoare, 1968; Bock, 1976). Additionally, inorganic substrates other than nitrite, namely nitric oxide, can be used for lithotrophic growth, indicating metabolic diversity among *Nitrobacter* species (Freitag et al., 1987). In anoxic environments, *Nitrobacter* cells are able to grow by denitrification (Freitag et al., 1987; Bock et al., 1988). Nitrate can be used as acceptor for electrons derived from organic compounds to promote anaerobic growth. Since the oxidation of nitrite is a reversible process, the nitrite oxidoreductase can reduce nitrate to nitrite in the absence of oxygen (Sundermeyer-Klinger et al., 1984). Furthermore, the nitrite oxidoreductase co-purifies with a nitrite reductase that reduces nitrite to nitric oxide (Ahlers et al., 1990).

Nitrite oxidation is a reversible process. The enzyme nitrite oxidoreductase (NO_2-OR) catalyzes the oxidation of nitrite to nitrate and the reduction of nitrate to nitrogen. The NO_2-OR is an inducible membrane protein present in the cells of *Nitrobacter*, which are either grown lithotrophically with nitrite or heterotrophically in the presence of nitrate. Depending upon the enzyme isolation technique, the molecular features of NO_2-OR vary considerably. Cytochromes of the *a*- and *c*-type were present when the enzyme of *Nitrobacter winogradskyi* was solubilized with Triton X-100 and purified by ion exchange and size exclusion chromatography (Tanaka et al., 1983).

In *Nitrobacter* species, absorption peaks at 605 nm in difference spectra indicate a cytochrome *c* oxidase of the aa_3-type. This membrane-bound enzyme was purified to an electrophoretically homogeneous state (Yamanaka et al., 1981; Sewell et al., 1972), and the function of cytochrome aa_3 was determined as a terminal oxidase by photoactivation of CO-inhibited oxygen consumption. In contrast to mitochondrial terminal oxidases, cytochrome aa_3 of *Nitrobacter winogradskyi* is composed of two subunits with 40 and 27 kDa in a molar ratio of 1:1 (Yamanaka et al., 1979). In *Nitrobacter vulgaris*, a membrane-bound nitrate reductase (NiR) was

co-purified with the nitrite oxidoreductase (Ahlers et al., 1990). The NiR reduces nitrite to nitric oxide, which is released under reduced oxygen partial pressure from the cells to the environment. Therefore, this enzyme seems to be a dissimilatory nitrite reductase of the denitrification type.

1.5.1.4 Heterotrophic nitrification

The oxidation of ammonia, hydroxylamine or organic nitrogen compounds, e.g., oximes (Castignetti and Hollocher, 1984), to nitrite and nitrate by various chemo-organotrophic microorganisms is called "heterotrophic nitrification." Heterotrophic nitrification is a cometabolism that is not coupled to energy conservation. Thus, growth of all heterotrophic nitrifiers is completely dependent on the oxidation of organic substrates (Focht and Verstraete, 1977; Kuenen and Robertson, 1987). The final product of heterotrophic nitrification often is nitrite (Castignetti and Gunner, 1980), so that heterotrophic nitrification may supply the substrate for lithotrophic nitrite oxidizers and heterotrophic denitrifiers. This additional nitrite production (together with the ability of nitrite oxidizers to grow chemo-organotrophically) might explain why in many environments the numbers of lithoautotrophic nitrite oxidizers are much higher than that of lithoautotrophic ammonia oxidizers (Kuenen and Robertson, 1987).

Many of the heterotrophic nitrifiers are capable of aerobic denitrification in the presence of organic matter, leading to the complete elimination of dissolved nitrogen compounds with the formation of gaseous nitrogen oxides and/or dinitrogen gas (Castignetti and Hollocher, 1984; Robertson et al., 1989;). Owing to the simultaneous nitrifying and denitrifying activity, nitrification rates of heterotrophic nitrifiers are often underestimated (Castignetti and Hollocher, 1984; Kuenen and Robertson, 1987). *Paracoccus denitrificans* (formerly called *Thiosphaera pantotropha*) produces nitrite from urea, ammonia and hydroxylamine and is also able to reduce nitrite even under aerobic conditions (Robertson and Kuenen, 1983; Robertson and Kuenen, 1984). Biochemically, the ammonia-oxidizing enzyme of *Paracoccus denitrificans* shows some similarities

to the AMO of lithotrophic ammonia oxidizers, e.g., the ability to oxidize alkanes, the apparent requirement for copper, and inhibition by light, diethyldithiocarbamate and allylthiourea (Moir et al., 1996; Crossmann et al., 1997). However, the genes encoding for these polypeptides are not closely related to the *amo*-genes of lithotrophic ammonia oxidizers (Crossmann et al., 1997).

The environmental importance of heterotrophic nitrifiers is controversial and generally it is assumed that in most environments, the biological conversion of reduced forms of nitrogen to nitrite and nitrate is catalyzed mainly by the lithoautotrophic ammonia- and nitrite-oxidizing bacteria and not by heterotrophic nitrifiers. This reflects that the nitrification rates of heterotrophic nitrifiers are small compared to those of autotrophic nitrifiers. Therefore, heterotrophic nitrification was thought to occur preferentially under conditions unfavorable for autotrophic nitrification, e.g., in acidic environments (Killham, 1986). In such environments, heterotrophic bacteria, fungi and even some algae might contribute considerably to nitrification. But according to some reports, even in acidic soils, heterotrophic nitrification contributes to overall nitrate production only to a minor extent (Barraclough and Puri, 1995).

1.5.1.5 Denitrification

Denitrification is a dissimilatory process of bacteria in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production. The gaseous end products NO, N_2O, and N_2 are released concomitantly. In the environment, denitrification is responsible for the release of fixed nitrogen into the atmosphere in form of N_2 (Knowles, 1982). It causes major nitrogen losses in agricultural soils to which fertilizers are applied. Accumulation of the greenhouse gases NO and N_2O leads to the destruction of the ozone layer (Conrad, 1996; Knowles, 1982). Also, denitrifying bacteria cause the removal of nitrogen compounds from waste water, where denitrification is coupled to the nitrification process (Knowles, 1982). Bioremediation of environmental pollutants can be achieved under denitrifying conditions (Fries et al., 1994; Hess et al., 1997).

The ability to denitrify is wide spread among bacteria of unrelated systematic affiliations, most likely due to lateral gene transfer in evolution. Denitrification is primarily heterotrophic, facultative, occurs under low oxygen conditions and is wide spread among over 50 different genera (Zumft, 1997), including members of Bacteria, Archaea, and based on the surprising discovery of complete denitrification in a benthic foraminifer -Eukarya (Risgaard-Petersen et al., 2006). Defined as a physiological group, these facultative anaerobes can switch from oxygen to nitrogen oxides as terminal electron acceptors when kept under anoxic conditions. Known denitrifying bacteria and archaea posses several clusters of genes involved in denitrification, and most are therefore capable of performing the multi-step process in its entirety ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$).

Nitrite reductase is the key enzyme in the dissimilatory denitrification process. The reduction of nitrite to NO can be catalyzed by the products of two different nitrite reductase genes: one product contains copper (the *nirK* product), and the other contains cytochrome *cd*1 (the *nirS* product). The two genes seem to occur mutually exclusively in a given strain, but both types have been found in different strains of the same species (Coyne, 1980). Although structurally different, both enzyme types are functionally and physiologically equivalent (Glockner et al., 1993; Zumft, 1997). *nirS* is more widely distributed; *nirK* is found in only 30% of the denitrifiers studied so far. However, *nirK* is found in a wider range of physiological groups (Coyne et al., 1989). Several different approaches were used to determine the type of nitrite reductase in laboratory pure cultures. Diethyldithiocarbamate has been used to identify *nirK*-containing denitrifiers (Shapleigh and Payne, 1985). Very specific detection, mostly at the strain level, could be achieved with antisera against dissimilatory nitrite reductase (dNirS Coyne *et al.*, 1989; Ward et al., 1993) and dNirK (Coyne et al., 1989; Michalski and Nicholas, 1988). Another approach was the use of gene probes for *nirK* (Kloos et al.,1995; Ye et al.,1992) or *nirS* (Kloos et al.,1995; Linne Von Berg et al., 1992; Ward et al., 1993), which were generally specific for the strains investigated. Weak reactivity also occurred for the *nirK* gene probe with DNA

from some of the other *nir*-type denitrifiers (Ye et al., 1992); the *nirS* probe, on the other hand, hybridized with a more limited variety of strains (Ward, 1995). A PCR method with one primer pair to target the *nirS* nitrite reductase gene showed higher specificity than hybridization experiments (Ward, 1995).

1.5.1.6 Denitrifying nitrifiers

Some ammonia-oxidizing *Nitrosomonas* spp. are capable of anaerobic growth on hydrogen by reducing NO_2^- to N_2 via the activities of nitrite and nitric oxide reductases (Bock, 1995; Schmidt et al., 2004). In addition, both *Nitrosomonas* and *Nitrosospira* spp. reduce NO_2^- and release substantial quantities of the potent greenhouse gas nitrous oxide (N_2O) under aerobic to suboxic conditions (Dundee and Hopkins, 2001; Shaw et al., 2005). Similarly, the nitrite-oxidizing *Nitrobacter* spp. produce N_2O during anaerobic respiration of NO_3^- and NO_2^- (Bock et al., 1988). Together, NO_2^- reduction by AOB and NOB, dubbed 'nitrifier denitrification', is thought to contribute as much, and perhaps more, N_2O to the atmosphere as heterotrophic denitrification in terrestrial (Webster and Hopkins, 1996) and marine ecosystems (Dore et al., 1998).

Ammonia oxidizers show relatively high denitrification activities when they are cultivated under oxygen-limited conditions in the presence of organic matter (mixotrophic growth conditions; Bock et al., 1995). However, under these conditions, ammonia oxidation rates are low (Zart et al., 1998). For this reason, the denitrifying potentials of ammonia oxidizers cannot be efficiently exploited for one-step nitrogen removal in wastewater treatment plants. In the absence of dissolved oxygen, *Nitrosomonas eutropha* and *Nitrosomonas europaea* are capable of anoxic denitrification using molecular hydrogen, or simple organic compounds such as acetate, pyruvate, or formate as electron donors and nitrite as electron acceptor (Bock et al., 1995; Abeliovich and Vonshak, 1992; Stüven et al., 1992).

In AOB, orthologues to *nirK* have been described in several marine *Nitrosomonas* species (Casciotti and Ward, 2001) and in the genome sequences of *Nitrosomonas*

europaea (Chain et al., 2003) and *Nitrosococcus oceani* (Klotz et al., 2006). Interestingly, a gene with high similarity to *nirK* in *N. europaea*, a Betaproteobacteria AOB, was found in the genome sequence of *Nitrobacter winogradskyi*, an Alphaproteobacteria NOB (Starkenburg et al., 2006). The *nirK* gene of *N. europaea* is located at the end of a four gene operon following a multicopper oxidase (*ncgA*) and two monoheme cytochrome *c* genes (*ncgBC*) (Beaumont et al., 2005), whereas the *nirK* gene of *N. winogradskyi* is located at the end of a five-gene operon of similar structure plus a cytochrome *c* biogenesis factor preceding *ncgA* (Starkenburg et al., 2006). In addition, both the *N. europaea* and *N. winogradskyi nirK* operons have a *nsrR* regulatory gene encoded upstream (divergently in *N. europaea* and convergently in *N. winogradskyi*) and binding motifs for NsrR in their promoter regions (Beaumont et al., 2004; Starkenburg et al., 2006). The presence of both the *nirK* operon and *nsrR* regulator in *Nitrosomonas* and *Nitrobacter* indicates an episode of horizontal gene transfer, suggesting a shared function between these phylogenetically distinct genera that form intimate associations with one another in the environment (Mobarry et al., 1996).

1.5.2 New Process and players in the nitrogen cycle

Much is now known about the nitrogen cycle and many of the micro-organisms involved, yet our understanding of the N cycle has been upended twice in past few years, first by the discovery of anaerobic ammonium oxidation (Anammox) in natural systems, and more recently by the discovery of aerobic ammonia oxidation within the domain Archaea (AOA). Understanding of these new processes and players in the microbial N cycle has evolved in opposite directions for anammox and the AOA. In the case of anammox, initial observations based on biogeochemistry led to the discovery of these organisms in the environment, and only now beginning to determine the biochemical pathways and genes involved in Anammox. AOA were first identified via functional gene sequences recovered directly from the environment, and still do not know what their full contributions to N biogeochemistry (Francis et al., 2007).

1.5.2.1 Anaerobic ammonium oxidizers (Anammox)

The existence of anaerobic ammonium oxidation (anammox) was hypothesized based on nutrient profiles and thermodynamic calculations (Broda, 1977; Richards, 1965; Strous and Jetten, 2004). It was first discovered (Mulder et al., 1995) in a pilot plant treating wastewater from a yeast-producing company in Delft, The Netherlands. The anammox reaction is the oxidation of ammonium under anoxic conditions with nitrite as the electron acceptor and dinitrogen gas as the product. Hydroxylamine and hydrazine were identified as important intermediates (Van de Graaf et al., 1996). Due to their very low growth rates (doubling time in enrichments is at best 11 days) the cultivation of the anammox bacteria proved to be tedious and required very efficient biomass retention (Strous et al., 1998). A physical purification of anammox organisms from enrichment cultures was achieved with percoll density centrifugation (Strous et al., 1999). The purified cells performed the anammox reaction after activation by hydrazine.

Survey of many wastewater treatment systems and freshwater ecosystems using anammox-specific probes and primers revealed the presence of significant populations of anammox bacteria. These bacteria belong to three genera : Candidatus "*Brocadia*" (Strous et al., 1999)., Candidatus "*Kuenenia*" (Egli et al., 2001; Fujii et al., 2002; Helmer-Madhok et al., 2002), and Candidatus "*Scalindua*" (Kuypers et al., 2003; Strous et al., 1999). Phylogenic analysis has shown that the three genera are monophyletic and branch off deep inside the planctomycete lineage of descent. All three genera share the same metabolism and have a similar ultrastructure. Thus, the capability for anammox has evolved only once. Still the evolutionary distances among the anammox genera are large; species of Candidatus "*Scalindua*" and Candidatus "*Brocadia*" on average have only 85% sequence identity in the 16S rDNA genes (Strous and Jetten, 2004).

Owing to their distinct metabolism and physiology, anammox bacteria received considerable attention in engineered systems, but were assumed to be minor players in the N cycle within natural ecosystems. However, nutrient profiles and

$_{15}$N tracer studies in suboxic marine and estuarine environments indicated that anammox is also a key player in the marine nitrogen cycle (Dalsgaard et al., 2003; Thamdrup and Dalsgaard, 2002; Trimmer et al., 2003). In addition, 16S rRNA gene analysis, fluorescence in situ hybridization (FISH), the distribution of specific anammox membrane lipids, nutrient profiles, and tracer experiments with [^{15}N]ammonia showed the link between the anammox reaction and the occurrence of the anammox bacterium Candidatus "*Scalindua sorokinii*" in the suboxic zone of the Black Sea (Kuypers et al., 2003). The anammox reaction has also been tested for implementation for full-scale removal of ammonia in wastewater treatment (Fux et al., 2002; Van Dongen et al., 2001; Van Loosdrecht et al., 2004).

1.5.2.2 Ammonia-oxidizing archaea (AOA)

Ammonia-oxidizing archaea (AOA) are among the most widely distributed and abundant groups of micro-organisms on the planet – the mesophilic Crenarchaeota. Although archaea were previously characterized as extremophiles, mesophilic archaea are now recognized to be an ubiquitous component of marine plankton (DeLong, 1992; Fuhrman et al., 1992), with the marine 'group 1' clade of Crenarchaeota alone comprising over 20% of picoplankton in the world ocean (Karner et al., 2001). These organisms are estimated to number a staggering 10^{28} cells in total; however, because of our inability to cultivate them, for the last 15 years our understanding of their physiology and biogeochemical function remained almost entirely speculative. Remarkably, two complementary metagenomic studies of seawater (Venter et al., 2004) and soil (Treusch et al., 2005) revealed putative ammonia mono-oxygenase (amoA) genes derived from uncultivated Crenarchaeota, suggesting the genetic capacity for ammonia oxidation. More specifically, Venter et al. identified an amoA-like gene on an archaeal-associated scaffold, whereas Treusch et al. found a similar gene on the same 43-kb soil DNA fragment as a 16S rRNA gene derived from a member of the group 1.1b Crenarchaeota – the most widespread crenarchaeal group in soils (Ochsenreiter et al., 2003).

The definitive link between these novel amoA genes and archaeal ammonia oxidation was recently and convincingly established by cultivation of an ammonia-oxidizing crenarchaeon – designated *Nitrosopumilus maritimus* – from a saltwater aquarium (Ko¨nneke et al., 2005). *N. maritimus* grows chemoautotrophically to cell densities of $10^7/mL^{-1}$ via the near-stoichiometric conversion of ammonia into nitrite, and with bicarbonate as a sole carbon source (organic carbon actually inhibited growth) (Konneke et al., 2005). 16S rRNA phylogeny places this organism firmly in the group 1.1a Crenarchaeota, the first cultivated representative from this exceptionally abundant archaeal group and it contains putative ammonia mono-oxygenase genes amoA, amoB and amoC. Molecular evidence demonstrating that the archaeal amoA gene to be pervasive in areas of the ocean that are critical for the global nitrogen cycle, including the base of the euphotic zone, suboxic water columns and coastal/estuarine sediments (Francis et al., 2005). Metagenomic analysis of the only other established species within the marine group 1.1a Crenarchaeota, the uncultivated sponge symbiont *Cenarchaeum symbiosum*, identified putative ammonia mono-oxygenase genes (amoA, amoB, amoC), as well as homologs of ammonia permease, urease, a urea transport system, putative nitrite reductase and nitric oxide reductase accessory protein, all potentially associated with chemoautotrophic ammonia oxidation (Hallam et al., 2006a). But homologs for critical components of the second enzymatic step of bacterial ammonia oxidation – hydroxylamine oxidoreductase and cytochromes c_{554} and c_{552} – were not identified (Hallam et al., 2006b). This has been interpreted by some as evidence that archaeal ammonia oxidation evolved fairly late by incorporating an AMO-like biochemical function into an ammonia-independent metabolism (Klotz et al., 2006). Recent findings suggest that most mesophilic Crenarchaeota are AOA, and that these organisms are the numerically dominant ammonia oxidizers in the ocean and in soils (Wuchter et al., 2006; Leininger et al., 2006). Adding to this emerging paradigm of AOA ubiquity, AOA have also recently been detected in nitrifying wastewater treatment bioreactors (Park et al., 2006). Closely related archaeal amoA sequences were recently recovered from an Austrian radioactive thermal spring (Weidler et al., 2007).

1.6 Processes of nitrogen removal in wastewater treatment

1.6.1 Sharon (single reactor high activity ammonia removal over nitrite) process

This is a partial nitrification process, the oxidation of wastewater ammonium to nitrite, but not to nitrate. To achieve partial nitrification, the subsequent oxidation of nitrite to nitrate must be prevented. In this process, both autotrophic nitrification and heterotrophic denitrification take place in a single Sharon reactor system using intermittent aeration The process needs less aeration, the subsequent denitrification consumes less COD (chemical oxygen demand), since only nitrite and not nitrate has to be reduced to molecular nitrogen (N_2) (Hellinga et al., 1998). The key step for controlling partial nitrification is to obtain a nitrification reactor with a stable nitrite accumulation. For this, different strategies and approaches have been used (Bernet et al., 2005), including the control of temperature, hydraulic retention time, the pH, dissolved oxygen in the reactor as well as the free ammonia. Temperature has different effects on the growth rate of ammonium and nitrite oxidizers. At temperatures above 25°C ammonium oxidizers can effectively out-compete the nitrite oxidizers (Brouwer and Van Loosdrecht, 1996; Van Dongen et al., 2001). If this condition is impaired with a low hydraulic retention time and also a low cellular retention time, nitrite oxidizers can be effectively washed out (Hellinga et al., 1998). This selective competition of nitrite oxidizers is the main concept of SHARON process. The denitrification (with added methanol) in the Sharon process is primarily required for pH control and alkalinity production, allowing completely for compensation of the acidifying effect in the nitrification phase. Although the process is not suitable for all wastewater due to a high temperature dependency, the Sharon process is ideally suited to remove nitrogen from waste streams with high ammonium concentration (>0.5 g/L) and temperature conditions.

1.6.2 Two- in- series sharon process - anammox process

Nitrogen compounds can be eliminated from ammonium rich wastewater by anaerobic ammonium oxidation (Anammox). However, ammonium in substrate must partly be preoxidized to nitrite (55–60% of ammonium), but not to nitrate, before

feeding into the Anammox process. Thus, the Anammox process needs to be applied by series operation with partial nitritation process such as a partial Sharon process. When the Sharon process is coupled with the Anammox process, the operational mode is changed to partial nitritation (conversion of only 55–60% of ammonium to nitrite) without heterotrophic denitrification, allowing adequate influent condition for the Anammox reaction. Generally, the partial nitritation process is performed in a single, stirred tank reactor with unique operating conditions: no sludge retention, about 1 day of HRT, 30–40°C of temperature and 6.6–7.0 of pH. This results in a stable nitrification with nitrite as the end-product (Jetten and Van Loosdrecht, 1997). Long-term nitrite production without nitrate accumulation in biofilm system can be unreliable because control of sludge age is difficult. In treatment of anaerobic digester effluent, no extra addition of base is necessary since the digester effluent generally contain enough alkalinity (in the form of bicarbonate) (Fux et al., 2004). Jetten et al. (1997) reported that in the partial Sharon–Anammox treating digester effluent, the overall nitrogen ammonium removal was 83% under a total nitrogen load of about 0.8 kg $N/m^3/day$.

1.6.3 Canon processes (completely autotrophic nitrogen removal over nitrite)

The concept of the Canon process is also the combination of partial nitritation and Anammox. However, this process performs two sequential reactions in a single and aerated reactor, implying that the two groups of bacteria (*Nitrosomonas*-like aerobic microorganisms and Planctomycete- like anaerobic bacteria) cooperate in the whole process (Third et al., 2002). Strous (2000) described that a co-culture of aerobic and anaerobic ammonium oxidizing bacteria could be established under oxygen- limited conditions. The nitrifiers oxidize ammonia to nitrite, consume oxygen and so create anoxic conditions the Anammox process need. The Canon process has quite sensitive operational characteristics in dissolved oxygen, nitrogen-surface load, biofilm thickness and temperature (Van Loosdrecht, 2004).

1.6.4 NOx process

The NO_x process is characterized by control and stimulation of denitrification activity of Nitrosomonas-like bacteria by adding trace amounts of nitrogen oxides (ratio of ammonium to nitrogen oxides = 1000–5000:1) into wastewater (Schmidt et al., 2001; Schmidt et al., 2002) Under fully oxic conditions, the supplemented NO_x (NO/NO_2) act as a regulator inducing the nitrification and denitrification activity of Nitrosomonas-like bacteria, simultaneously (Schmidt et al., 2002). This new process was tested in laboratory and pilot scale nitrification plants. In the operation of 3.5 m^3 pilot scale plant treating highly loaded sludge liquor (about 2 kg NH_4-N/m^3) with 200 ppm NO_2, average nitrogen loss to N_2 gas was about 67%. Strong evidence was presented that ammonium oxidizer was mainly responsible for nitrogen loss. The operating results also demonstrated that the denitrification activity of the nitrifying biomass was very sensitive towards the NO_2 supply.

1.6.5 Oland process (oxygen-limited autotrophic nitrification and denitrification)

The OLAND process (oxygen-limited nitrification and denitrification) is described as a new process for one-step ammonium removal without addition of COD (Kuai and Verstraete, 1998). OLAND will be based on either the Canon concept (a combination of aerobic and anaerobic ammonia oxidizers) or the NO_x process (nitrifier denitrification in the presence of NO_x). The main difference between this process and the Canon process is that OLAND make use of the denitrification activity of conventional aerobic nitrifiers, whereas the Canon incorporates the Anammox process. These processes have been tested on pilot and full-scale plants treating ammonium rich wastewater. Detailed mechanism on these processes is not well understood yet. The nitrogen loading and conversion are quite low (Seyfried et al., 2001).

1.6.6 Deamox process

A variation of the Anammox process was tested recently on a laboratory scale and was called Deamox (Denitrifying ammonium oxidation). This process is based on the combination of the Anammox reaction under autotrophic denitrifying conditions using sulfide as an electron donor for the production of nitrite from

nitate within an anaerobic biofilm (Kalyuzhnyi et al., 2006). Further investigation of this concept is needed. The presence of hydrogen sulfide may provide inhibition on all bacterial species involved. In addition, this concept may only be limited to sulfate containing wastewaters (Kalyuzhnyi et al., 2006).

Even though so many new processes developed, applying them into the aquaculture system has got many limitations and still follows the conventional nitrification using the chemolithoautotrophs and heterotrophic denitrification. Compared with domestic wastewater (Metcalf and Eddy Inc., 1991; Henze et al., 1997), aquaculture wastewater has a relatively low concentration of pollutants (Piedrahita, 2003), and thus, bacterial biomass yield in treatment systems is also low. To treat this type of water, bioreactors with a high bacterial cell residence time are required (Bovendeur, 1989).

1.6.7 Nitrification in aquaculture: biofilters

Biological nitrification can be accomplished in two types of systems: suspended and attached growth. Under a suspended growth environment, the microorganism is freely mobile in the liquid providing direct contact between the bacteria cells and the bulk water. In an attached growth system, on the other hand, microorganisms are grown in a viscoelastic layer of biofilm that are attached on the surface of a solid support medium. Thus, this process is also called a fixed film process in which the individual bacteria are immobilized. Attached growth on a fixed biofilm system offers several advantages when compared to suspended growth processes, such as handling convenience, increasing process stability in terms of withstanding shock loading and preventing the bacteria population from being washed off (Fitch et al., 1998; Nogueira et al., 1998). Because most aquatic species tolerate only low level ammonia concentrations, which is most suitable for the advantages of attached growth processes, biofilters that operate on the principle of attached biofilm have been extensively applied in RAS. In a fixed film biological process, the dissolved or colloidal wastes are transported by diffusion into the biofilm, which coats a filter media. Rock, shells, sand and plastic are commonly used to support these bacterial

films. The biofilm can be viewed as a bacterial habitat that endures wide varieties of flow and quality regimes while maintaining its inherent ability to process wastes (Wheaton, 1977). Biological filters are widely used for freshwater and marine operations (Hovanec and Delong, 1996; Gutierrez-Wing and Malone, 2006; Malone and Pfeiffer, 2006).

Nitrification is carried out in a variety of systems, which can be grouped into two general types: emerged (rotating biological contactors, trickling filters) and submerged (eg. Fluidized bed filters, bead filters) fixed film filters (van Rijn, 1996; Ling and Chen, 2005; Malone and Pfeiffer, 2006). Fixed film biofilters can be organized into four fundamental blocks distinguished by the strategy used to provide oxygen, and by their means of handling biofilm growth (Fig. 3).

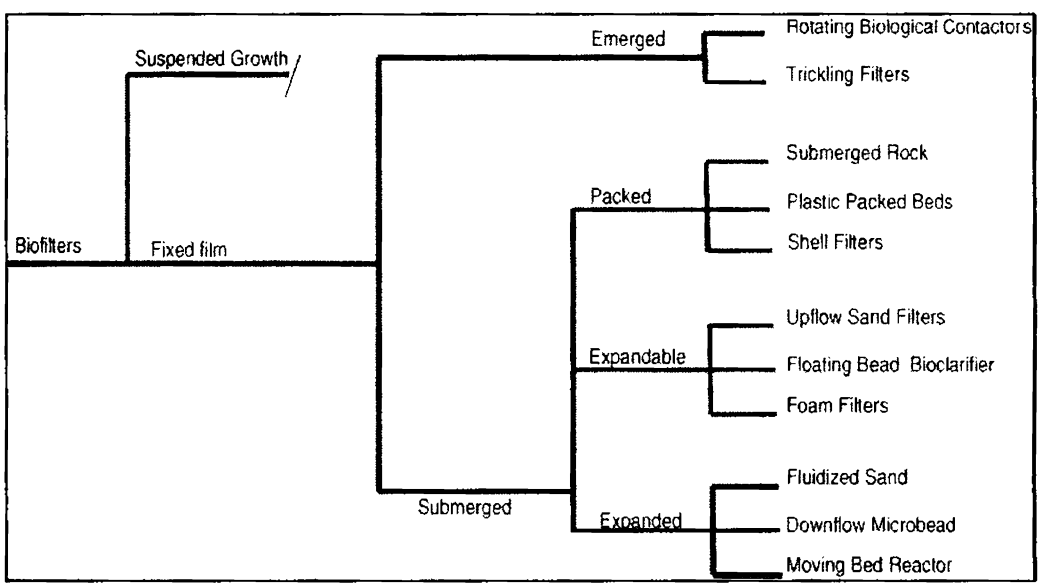

Fig.3. RAS biofilters clustered into four basic blocks that display similar characteristics (Malone and Pfeiffer, 2006)

1.7 Emergent filters

The "emergent" filters are designed to maximize oxygen transfer as water cascades directly over the media. In the case of the tricking filter the cascade is achieved by water falling over the media (Twarowska et al., 1997; Greiner and Timmons, 1998; Lekang and Kleppe, 2000; Eding and Kamstra, 2001; Sandu et

al., 2002; Shnel et al., 2002), whereas, rotating biological contactors create the same effect by rotating the media in and out of the water (De Los Reyes and Lawson, 1996). The media is fixed and biofilm accumulations are usually managed through the process of sloughing. The sloughing process demands a relatively high porosity (as well as high interstitial distances) so released biofilm can fall free of the media. Thus, to avoid biofouling problems, emergent filters are associated with media displaying relatively low specific surface areas. Rotating biological contactors have been used in the treatment of domestic wastewater for decades and are now widely used as nitrifying filters in aquaculture application. Rotating biological contactor technology is based on the rotation of a submerged substrate, which is made of high-density polystyrene or polyvinyl chloride, attached to a shaft (Tawick et al., 2004; Park et al., 2005; Brazil, 2006). Biodrums, a variant of the RBC utilizes a media held loosely in an enclosed rotating drum (Wortman and Wheaton, 1991) to enhance biofilm removal capabilities while maintaining the secondary benefits of aeration and CO_2 stripping provided by the cascading water displayed by all the emergent designs (Hall, 1999). Trickling filters consists of a fixed medium bed through which aquaculture wastewater flows downwards over a thin aerobic biofilm (Eding et al., 2006). Trickling medium has a specific surface area ranging from 100 to 1000 m^2/ m^3. Finturf artificial grass (284 m^2/m^3), Kaldnes rings (500m^2/ m^3), Norton rings (220 m^2/m^3) and Leca or light weight clay aggregate (500-1000 m^2/m^3) are some of the most frequently used media (Greiner and Timmons, 1998; Lekang and Kleppe, 2000; Timmons et al ., 2006). Kamstra et al. (1998) reported TAN areal removal rates between 0.24 and 0.55 g TAN/m^2 day for a commercial- scale trickling filter. For three different applied filter medium types in commercial farms and a range of hydraulic surface loading conditions, the highest observed TAN areal removal rate for a trickling filter was 1.1g TAN/m^2 day, with an average TAN areal removal rate of about 0.16 g TAN/ m^2 day (Schnel et al., 2002; Eding et al., 2006). Lyssenko and Wheaton (2006) reported TAN areal removal rates of 0.64 g TAN/m^2 day.

1.7.1 Submerged filters

Submerged filters presume that sufficient oxygen can be transported with waters circulated through the filter. This presumption is normally assured by use of high recirculation rates, internal recycling, or through oxygen enrichment of influent waters. These filters are distinguished by the strategies used to manage their biofilm accumulations. There are three fundamental types of submerged filters. The first category utilizes static "packed" beds that provide no active means of biofilm or solids removal. Submerged rock filters and submerged packed beds depend entirely upon endogenous respiration to control biofilm accumulations (Manthe et al., 1988). Water flow may be from top to bottom (downflow) or from bottom up (upflow). These filters are used mainly in lightly loaded systems such as display aquaria or crab shedding systems. They have, however, also been utilized in a variety of formats for recirculating shrimp production systems that are more heavily loaded (Davis and Arnold, 1998; Tseng et al., 1998). They are inexpensive and work well until overfeeding and solids accumulation in the packed bed causes excessive bacterial growth, which limits water penetration. Some variants utilize blown air or draining to mitigate the solids accumulation issue.

The second category of submerged beds employs static beds that are intermittently "expandable". Biofilm grown while the bed is static is periodically removed by the process of abrasion as the individual medium elements move randomly rubbing or striking each other. Motion in the bed is induced by hydraulic, pneumatic or mechanical forces. The aggression of the backwashing mechanism and backwash frequency are employed to control the biofilm growth (Sastry et al., 1999; Golz et al., 1999; Tijhuis et al., 1996). The ability to clean the media allows the use of a much smaller media than used in the packed bed formats. These approaches have been limited to coarse sands or larger beads that have the mass capable of generating the momentum necessary during a cleaning event of short duration. The inability to maximize specific surface area is offset by the management flexibility provided by the backwashing strategy and these filters

usually show the ability to be used concurrently as solid capture devices (Cooley, 1979). Custom bead designs can also be used to overcome the specific surface area shortcomings (Beecher et al., 1997). Sponge filters are typically small but a widely recognized variant of this strategy. Backwashing in this case is provided by manual compression of the media.

The third category of submerged filters abandons the static bed in favor to a medium that is in constant motion. "Expanded" filters continually abrade biofilm by a hydraulic or pneumatic means. The rate of biofilm loss is usually determined by the media selection. These units can utilize granular media with extremely high specific surface areas, although the finest media are limited in their abrasion capabilities and thus find limited use in eutrophic applications. Fluidized filters are the oldest of this class. Fluidized beds can incorporate diverse material types (granite, anthracite, activated carbon) and a wide variety of particulate diameters as a means of adjusting the abrasion requirements to the trophic level of the application (Heinen et al., 1996). These fluidized beds are typically expanded by hydraulic means although a number of units have been augmented by pneumatic means. Fluidized sand biofilters have been widely adopted in recirculating systems that must reliably maintain excellent water quality. Filter sand has a high specific surface area, i.e. 4000-20000 m^2/ m^3 and has a moderate cost (Summerfelt, 2006). A disadvantage of the FBS is that they do not aerate, as do trickling filters. Therefore, additional aeration is needed. These filters also must operate within a narrow water flow range in order to maintain proper bed expansion (Summerfelt, 2006). Miller and Libey (1986) demonstrated that the TAN removal rate of a fluidized bed reactor was around 0.24g $N/m^2/day$. Timmons and Summerfelt (1998) found similar rates in their research. A second subclass of filters employs a limited degree of media movement to maintain the hydraulic conductivity of the media. Microbead filters employ this strategy to achieve conversion across a variety of trophic levels (Greiner and Timmons, 1998). These filters use a small floating polystyrene bead in a downflow mode to take advantage of high specific area characteristics of the media. Moving bead

reactors show promise as they operate across a wide range of substrate concentration, display excellent TAN conversion rates, and can operate in a low-head environment (Zhu and Chen, 1999). This emerging technology employs aeration to control biofilm development in a larger media that mitigates excessive abrasion by providing protected areas for biofilm development (Odegaard et al., 1994). Seo et al. (2001) describes a similar approach that employs immobilized nitrifying bacteria.

1.8 Factors affecting nitrifying biofilm kinetics of biofilters

Various types of fixed film biofilters have been used in recirculating aquaculture systems under different water quality and operating conditions. The effectiveness of the nitrification process can be evaluated by nitrification kinetics. There are more than 20 physical, chemical and biological factors that can affect the growth or the substrate supply, thus ultimately influencing the performance of nitrification biofilters (Wheaton et al., 1994). Most significant factors can be classified into three major categories. The first category includes those that affect the biochemical process of the microbes such as pH, temperature and salinity. The second category includes those that affect the supply of nutrients to the biofilm such as substrate concentration, dissolved oxygen (DO) and mixing regime. The third category includes those that have impact upon both growth and nutrient supplies, such as the competition for either essential nutrients or space, represented by the level of organics (Chen et al., 2006). The impacts of these parameters upon nitrification kinetics make predicting the performance of a biofilter for a given application an engineering challenge. Knowing the performance of a biofilter is critical for both designers and managers (Chen et al., 2006).

The kinetics of biofilm is more complex, as the substrate supply into the layer-like aggregation of bacteria film is a diffusion-controlled process driven by the concentration gradient across the biofilm. Next to the biofilm there is a water film that serves as the interface between the biofilm and the bulk water. Therefore, the kinetics of biofilm reactions is influenced by mass transport (Rasmussen and

Lewandowski, 1998). Factors that determine diffusion rate, such as the local chemical environment and flow conditions, influence the rate of substrate supply and subsequently, the extent of biofilm growth. The diffusion and transport process should be considered in order to better understand the nitrification kinetics of fixed film biofilters. It was reported that liquid film diffusion has a considerable influence on the performance of a fixed film reactor (Tanaka and Dunn, 1982). Since nitrification reactions occur in the biofilm instead of in the bulk liquid (Moreau et al., 1994), the substrate utilization rate depends on local substrate concentrations within the biofilm. Nitrifying populations deep within the biofilm are maintained by endogenous respiration under limited oxygen conditions, and nitrifying populations on the surface are the only survivors under limited ammonia conditions (Horn, 1994). At local reaction sites, reactant concentrations are depressed, and product concentrations are elevated. Therefore, substrate concentration is different at the different depths of the biofilm (Boller et al., 1994). A precise analysis of nitrification kinetics as a function of substrate concentration has to consider the variation in substrate concentrations within the biofilm. However, for simplicity, attempts have been made to relate the nitrification rate directly to total ammonia concentration in the bulk liquid (Zhu and Chen, 1999).

The nitrification rate in a biofilm is in equilibrium between substrate demand created by the growth of bacterial biomass and the rate of substrate supply determined by diffusion transport limitation (Rasmussen and Lewandowski, 1998). The substrate demand is determined by the factors that are related to the characteristics of nitrifiers such as the amount of nitrifier biomass, the specific growth rate and yield coefficient. The substrate supply is determined by the diffusion rate of essential nutrients. The concentration of TAN as the substrate of nitrification is the most important factor to consider in biofilter design and operation. In general, nitrification biofilters used in a RAS should be designed based on ammonia concentration (Wheaton et al., 1994). The biofilters must be able to maintain a high quality of water with sufficiently low ammonia

concentration and these biofilters must process TAN at an adequate rate, at a given level, to prevent TAN accumulation. The concept of a minimum substrate concentration required to support a steady state biofilm was proposed and proven by Rittmann and McCarty (1980) and Rittmann and Manem (1992). Substrate inhibitions on nitrification biofilter efficiency were reported with excessively high ammonia or nitrite concentrations (Sharma and Ahlert, 1977; Carrera et al., 2004). However, the substrate limitation rather than substrate inhibition is often the major concern for biofilter designs in RAS due to the low ammonia concentration in these systems (Wheaton et al., 1994).

For RAS design, knowledge of the relationship between nitrification rate and DO concentration is a major interest. DO of 2 mg/L was suggested as the minimum oxygen level in aquaculture nitrification biofilters (Wheaton et al., 1994). Zhu and Chen (2002) reported that it was more important to maintain sufficient DO in the fixed film process than in the suspended growth processes due to the nature of diffusion transport with fixed film. Low DO concentrations can also cause nitrite accumulation in nitrification biofilters. Turbulence affects the thickness of the water film and subsequently the transfer resistance of substrate from bulk liquid into the biofilm. Therefore, turbulence has great impact on the mass transfer flux into biofilm as well as the nitrification rate (Chen et al., 2006).

The most important impact of organics upon nitrification is due to the contribution of additional oxygen demand. Particulate and dissolved organics provide substrates for heterotrophic bacteria whose growth will compete with nitrifying bacteria for oxygen and growing space (Ohashi et al., 1995; van Benthum et al., 1997). With the addition of organic matter, fast-growing heterotrophic bacteria which use organic carbon as their energy source will out compete slow-growing nitrifying bacteria, resulting in a decrease in the nitrification rate (Grady and Lim, 1980). Higher influent C/N ratio retarded accumulation of nitrifying bacteria and resulted in a considerably longer start-up period for nitrification. It was also reported that the regulation of nitrification by organic carbon was not only

dependent on the quantity but also the quality of organic carbon, where higher quality organic carbon had a stronger negative impact on nitrification (Strauss and Lamberti, 2000). Higher temperature enhances nitrification rate as the biochemical driven bacterial processes accelerate as temperature increases. However, the impacts of temperature change on nitrification rate in fixed film biofilters were poorly understood (Okey and Albertson, 1989). Little information is available to quantify the effects of temperature on fixed film nitrification rate (Wheaton et al., 1994).

According to Villaverde et al. (1997), the optimal range of pH for nitrification can be determined by the three different effects that the pH can exert on nitrifying bacteria: (1) activation–deactivation of nitrifying bacteria; (2) nutritional effect, connected with alkalinity; (3) inhibition through free ammonia and free nitrous acid. Substantial changes in the pH across the boundary layer and the biofilm were also predicted by using a steady-state complexity nitrifying biofilm model and considering both pH effects and ion interactions (Flora et al., 1999).Alkalinity in the form of carbonate and bicarbonate is a nutrient element for nitrifying bacteria. In addition, alkalinity provides the buffering capacity that is necessary to prevent pH changes due to acid production in the nitrification process. Therefore, the impact of alkalinity on the nitrification rate is also related to that of the pH. Nitrification efficiency showed a linear increase of 13% per unit pH increase from pH 5.0 to 8.5 and observed a linear correlation between the alkalinity (as mg $CaCO_3$ /L) and pH, with a stoichiometry coefficient of 7.1 mg $CaCO_3$ consumed/ mg NH_4 -N oxidized (Villaverde et al., 1997). Relatively limited information is available concerning the effect of salinity on nitrification kinetics. There are discrepancies in the reports, probably due to different experimental conditions. Nijhof and Bovendeur (1990) compared the nitrification characteristics of salt water RAS with that of fresh water systems. The results indicated that the maximum nitrification capacity in the salt water systems was considerably lower than in fresh water systems.

1.9 Modeling nitrification process in aquaculture

Although fixed-film bioreactors have been well adopted in wastewater treatment and recirculating aquaculture systems (RAS) for the removal of ammonia nitrogen, design is often based on empirical data or trial-and-error experimentation under particular conditions (Rittmann and Stilwell, 2002). When empirical data is lacking or operating conditions change, biofilters are usually oversized or undersized for the system leading to either a waste of energy and material resources or system failure. Employing a mathematical model as a design tool has been suggested to overcome these problems and improve the efficiency of the biofilters. The early biofilm models were based on the conventional conceptual model with a homogenous biofilm structure, which consisted of a base film and a surface film (Characklis and Marshall, 1990). In this concept, the transport of substrates, nutrients, and products in and out of the biofilm is by molecular diffusion only. Transport between the bulk fluid and the biofilm, on the other hand, is dominated by advection and turbulent diffusion (Grady et al., 1999). Mathematical models based on this conceptual model were mostly one-dimensional (1D) with the assumption of an evenly distributed biofilm structure and biomass and usually only the base film was considered (Noguera et al., 1999). Most of the early biofilm models only considered single species biofilm to simplify the computation with analytical solutions. Although some of the biofilm models might have considered competitions with mixed culture they either required complex numerical solutions (AQUASIM, Wanner and Morgenroth, 2004) or were associated with limiting-case conditions to obtain model solutions.

Therefore, two-dimensional (2D) and three-dimensional (3D) models have been worked on using approaches such as "biomass-based modeling" and "individual-based modeling" (Noguera et al., 1999; Xavier et al., 2004). However, it is currently difficult to apply the microscale information provided by the complex 3D model to a macroscale level due to the lack of experimental data on the behavior of individual cells as well as on the availability of their kinetic

parameters (Wanner, 2002). Many biofilm models have concentrated mainly on describing organism populations that makes today's models less relevant to the real world applications, especially for engineering practices (Ling, 2005). From a practitioner perspective, the physical operation of the reactor may have a greater impact on the systems performance than population dynamics and micro-scale transport processes in the biofilm. Moreover, with too many details on microscale biofilms included, models may not be able to reproduce the behavior of a full-scale biofilm reactor (Morgenroth et al., 2000). Some of the input parameters may not be measurable, which makes calibrations impossible for many mathematical biofilm models. Finally, the expensive computation cost also limits the applications of multi-dimensional models. It is impractical to apply multi-dimensional biofilm models in the design of biofilters for wastewater treatment and RAS at the present time (Ling, 2005).

For application in hatchery conditions, the nitrification rate of a bioreactor can be tested in terms of substrate utilization. Monod relationships are used to describe the relationship between the rates of substrate utilization. The Monod kinetics is underpinned by Michaelis- Menton enzyme kinetics (Knowles et al., 1965; Malone et al., 1993) which presumes substrate limitation within the context of an enzymatic reaction. Nitrification is normally controlled by diffusion of TAN through a water boundary layer and through the biofilm itself, a phenomenon that has been shown to be well represented by Monod type kinetics (Tanaka and Dunn, 1982; Harremoes, 1982). The impacts of diffusional characteristics on nitrite conversion are not as clearly defined as nitrite is generated and converted within the biofilm (Schramm et al., 1996). This kinetic approach projects filter performance in both the linear (low substrate) and zero order (high substrate) concentration regimes (Malone and Pfeiffer, 2006). The principle shortcoming stems from the fact that neither of the fixed parameters can be realistically expected to be constant across differing organic carbon loading regimes. Therefore, a simple and practical approach would be to model the experimental

biofilter systems using Monod kinetic equations to understand and predict its flexibility and efficiency with change in substrate concentrations.

1.10 Nitrifying bioreactors for tropical hatchery systems

There are a wide variety of commercially available biofilters and biofilter media on the market today. In its most basic form, all biofilters perform the same elementary task of removing total ammonia nitrogen (TAN) from water. Additionally, biofilters are typically sized according to either the volume of media (m^3) they contain or the specific surface area (m^2/m^3 of media) of the media used (Drennal II et al., 2006). The basic design criterion for biofilters is the diurnal waste production and total ammonia nitrogen load in to a system which varies with the cultured species and biomass load into the system. The quality and the quantity of the waste are dependent on fish and feed related aspects (Heinsbroek, 1988). When a recirculation system is designed, commonly published data on waste production must be validated for the specific conditions of the designed production system (Eding et al., 2006).

However, most of the biofilter systems developed and validated are for application in temperate aquaculture systems, which may not function effectively under significantly different environmental conditions of the tropics. Moreover, in the tropics aquaculture is a livelihood for the people, and the design of cost-effective bioflilters is a more pragmatic approach than adopting existing technologies from the market. Therefore, it became imperative for the tropics to develop user-friendly and economically viable technologies having the advantages of short start-up time and easiness to integrate to the existing hatchery designs without modifications. Accordingly, two specialized nitrifying bioreactors were developed with indigenous nitrifying bacterial consortia and tested and proved for potential for nitrification (Achuthan, 2000 and Anon, 2002). The *ex situ* packed bed bioreactor (PBBR) serves the purpose of reuse and recycling of spent water for larval production, maturation systems and for the treatment of fresh seawater. The *in situ* Stringed Bed Suspended Bioreactor (SBSBR) is deployed in the

culture systems for in situ nitrification especially in the larval rearing tanks. These reactors (PCT Patent application no. 828/DEL/2000/India), contain specially designed polystyrene and polyethelyene beads as the substrata for immobilizing nitrifying bacterial consortia. Two ammonia oxidizing consortia such AMOPCU-1 (Ammonia oxidizers for penaeid culture)and AMONPCU-1 (Ammonia oxidizers for non-penaeid culture) and two nitrite oxidizing consortia such as NIOPCU-1 (Nitrite oxidizers for penaeid culture) and NIONPCU-1 (Nitrite oxidizers for non-penaeid culture) developed under constant salinity regimes from brackish water environments were used for activating the reactors (Achuthan, 2000). For wide adoption of the technology in the market, it required further optimization, fine tuning and validation at pilot to field levels; the consortia required resolution of community and the present study is focused in this direction

1.11 Objectives

1. Molecular characterization of the nitrifying bacterial consortia
2. Development of mass production process for the nitrifying bacterial consortia
3. Activation of the reactors with the mass produced consortia, nitrification kinetics of the reactor and modeling
4. Validation of the reactors at recirculating aquaculture systems

Chapter 2

MOLECULAR CHARACTERIZATION OF THE NITRIFYING BACTERIAL CONSORTIA

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  2.3.1. Ultrastructure of the consortia
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2.1 Introduction

Optimizing the parameters for biological nitrogen removal in recirculating aquaculture systems is an important research area in the recent times. Despite their importance, there is a dearth of information about the identity and ecology of the microorganisms involved in the process (van Rijn, 1996). Most studies on N-removing bacteria in recirculation aquaculture filters or wastewater treatment plants have focused on the nitrifying consortia belonging to the β-and α-subdivisions of the Proteobacteria, i.e. *Nitrosomonas* sp., *Nitrobacter* sp., and *Nitrospira* sp., that occupy aerobic biofiltration units (Juretschko et al., 1998; Princic et al., 1998; Kloep et al., 2000). For many years, the general assumption has been that ammonia and nitrite-oxidizing species were identical in marine and freshwater environments. Recent innovations in microbial ecology techniques, however, have shown that this is not the case. Using oligonucleotide probes Hovanec and Delong (1996) demonstrated *Nitrosomonas europaea* the bacteria responsible for ammonia oxidation, to be present both in seawater as well as in freshwater aquaria. Other studies showed that the important nitrite-oxidizing bacteria in fresh and marine environments belonged to *Nitrospira* sp. and not to *Nitrobacter* sp. as was previously thought off (Burrell et al., 1998; Daims et al., 2000). While a few studies have begun to examine biological filtration systems used in aquaculture, almost no information is available about the specific members that compose the bacterial consortia in nitrifying filters and the role that they play in N-removal processes. For instance, the filters often contain anaerobic regions and the involvement of anaerobic ammonia oxidizing (anammox) bacteria (Strous et al., 1999) in contributing to ammonia removal has not been examined. The wide range of physical and chemical conditions present in these biofilters as well as the various nutrients that are available for bacterial consumption makes it likely that many key microorganisms have not been reported. One of the main problems in exploring microbial diversity in complex environments, such as those associated with biological filters, is the fact that many of the important microorganisms cannot be cultivated by traditional methods (Amann et al., 1995).

Identification, enumeration and characterization of ammonia- and nitrite-oxidizing bacteria in environmental samples by traditional microscopical and microbiological methods are difficult, because of the limited species specific morphological variety, slow growth rate (Watson et al., 1989), and their low growth yields (Gay and Corman, 1984; Wood, 1986). The use of molecular techniques to study nitrifying bacteria now enables us to circumvent these limitations, and to obtain useful information on their phylogenetic relationships, and their ecological significance.

The availability of molecular tools, such as those used to analyze 16S ribosomal RNA (rRNA) sequences, have made it possible to explore slow growing or uncultivated bacterial species in different environments (Heal et al., 1998; O'Donnell and Gorres, 1999).

The oldest among the molecular approaches to detect and identify ammonia- and nitrite-oxidizing bacteria is the immunological approach. Specific antibodies have been used in immunofluorescence assays to study nitrifiers in soils (Belser and Schmidt, 1978; Fliermans et al., 1974: Josserand and Cleyet-Marel, 1979), wastewater (Yoshioka et al., 1982), and seawater (Ward and Perry 1980; Ward 1982; Ward & Carlucci 1985). Sanden et al., (1994) used monoclonal antibodies specific for *Nitrosomonas* and *Nitrobacter* in a competitive enzyme-linked immunosorbent assay (ELISA) to characterize and quantify these bacteria in activated sludge from wastewater treatment plants. Monoclonal antibodies developed by Bartosch et al., 1999 recognizing the nitrite oxidoreductase can be used for the identification of nitrite-oxidizing bacteria. Polyclonal antibodies recognizing the amoB protein of ammonia oxidizers were developed for the detection of ammonia oxidizers of the β-subclass of the Proteobacteria (Pinck et al., 2001) However, although successful, the immunological approach is hampered by the need of pure strains to produce antisera.

The ribosomal RNA approach, initiated by Pace and coworkers (Olsen et al., 1986), and the application of new molecular biological techniques, such as PCR (Saiki et al., 1988), and fluorescence in situ hybridization (FISH; Amann et al.,

1995), are gaining importance to study the structure and function of microbial communities, and the role of individual inhabitants, such as the nitrifiers, since these techniques do not directly depend on the need of isolated strains (Muyzer and Ramsing, 1996).

The fact that nitrifying bacteria (ammonia and nitrite oxidizers) seem to have only one operon for 16S rRNA genes (Navarro et al., 1992; Aakra et al., 1999) greatly facilitates community analyses by molecular approaches. The knowledge of these sequences allows different approaches for studying the community composition of nitrifying bacteria. Most of the work has been done for the monophyletic group of the AOB within the β-Proteobacteria. The monophyletic nature of ammonia-oxidizing baceria (AOB) allows rapid development of molecular tools such as PCR primers and hybridization probes (McCaig et al., 1994; Mobarry et al., 1996). By using comparative analysis of the determined 16S rRNA sequences, McCaig et al. (1994), and Voytek and Ward (1995) were able to design specific primers for amplification of the 16S rRNA gene of ammonia oxidizers belonging to the ß-subgroup of Proteobacteria. McCaig et al. (1994) used this specific amplification approach as an early molecular characterization of ammonia oxidizers in enrichment cultures obtained from surface seawater. After sequence analysis of the PCR fragments three new lineages of β-ammonium oxidizing bacteria were obtained. Voytek and Ward (1995) used their specific primer set for the detection of ammonium-oxidizing bacteria in aquatic samples without any prior cultivation step.

Apart from using rRNA or their encoding genes as molecular marker, functional genes too have been used to detect nitrifiers in their habitat. The genes encoding for ammonia monoxygenase has been used as an alternative target for the molecular analysis of the ammonia oxidizing bacteria (Sinigalliano et al.,1995; Rotthauwe et al., 1997; Juretschko et al., 1998; Norton et al., 2002). Calvo and Garcia-Gil (2004) developed primers for the amplification of the amoB subunit of

the ammonia monoxygenase and suggested its use as a molecular marker for ammonia–oxidizing bacteria.

Both *amoA* and 16S rRNA genes have become targets of quantitative PCR (qPCR) using the internal standard approach (competitive PCR, cPCR) for quantification of β subclass AOB in environmental samples (Mendum et al., 1999; Stephen et al., 1999; Phillips et al., 2000). The targeting of 16S rRNA has one advantage over *amoA*, in that all known β subclass AOB are thought to carry only a single 16S rRNA gene per genome, whereas *amoA* copy number may vary (usually either 2 or 3). However, none of the 16S rRNA gene primer sets designed to be specific to the entire β subclass AOB radiation are 100% specific in all samples (Juretschko et al., 1998; Kowalchuk et al., 1998). Therefore, use of 16S rDNA as a target for cPCR demands that the PCR products generated from a given sample lack sequences derived from co-amplifiable non-AOB source organisms. Some of the primer pairs targeting *amoA* are totally specific and apparently more sensitive than those targeting 16S rRNA (Kowalchuk et al., 1999; Mendum et al., 1999). However, given our limited knowledge of the full diversity of *amoA* genes, it is difficult to predict the coverage of *amoA*-directed cPCR strategies.

Aakra et al. (1999) used the ribosomal internal transcribed spacer regions (ITS) to the characterization of several closely related *Nitrosospira* strains and compared ITS results with those obtained by 16S rDNA sequencing. Aoi et al. (2004) applied real-time reverse transcription PCR analysis to investigate the response of amoA mRNA and rRNA levels to a change in ammonia concentration which manage cell activity in batch mode incubation, and the relationship between amoA mRNA level and ammonia-oxidation activity in a continuously fed nitrification reaction. Smit et al. (1997) used the amplified ribosomal DNA restriction analysis (ARDRA) of PCR-amplified 16S rRNA gene fragments for detecting the copper-related changes in the AOB community composition.

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes is a cultivation-independent technique that allows visualization of bacteria (or other microorganisms) specifically and directly in their habitats (DeLong et al., 1989; Amann, 1995; Amann et al., 1995). The oligonucleotide probes are specific for single species, or whole genera, or even phyla and domains according to the sequence conservation at their target sites on the rRNA (Amann et al., 1995). FISH with rRNA-targeted probes can be combined effectively with comparative rRNA sequence analysis: A first overview of the bacterial community composition in an environmental sample is obtained by hybridization of the sample with existing probes that target different phylogenetic groups of bacteria. In parallel, rRNA gene libraries of the sample are established and screened for sequences of new or otherwise interesting bacteria. Based on these rRNA sequences, new probes are developed which detect the corresponding organisms in situ. This "rRNA approach" (Amann et al., 1995) proved to be highly useful for investigating microbial communities in numerous different, natural and artificial habitats. Up to seven different populations could be detected in the same experiment when several oligonucleotide probes, labeled with different fluorochromes, were applied simultaneously (Amann et al., 1996).

Comparative analyses of 16S rRNA sequences were used to design probes for in situ detection of ammonia-oxidizing bacteria (Wagner et al., 1995), and for the study of nitrifiers in multi-species biofilms (Ohashi et al., 1996). By the combined use of a fluorescent-labeled oligonucleotide probe specific for ammonia-oxidizing bacteria and confocal scanning laser microscopy, Wagner et al. (1995) were able to detect dense cell clusters in samples of sewage treatment plants with stable nitrification. No fluorescent cells were detected in samples without nitrification. Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes have been used by various researchers for studying the population structure and changes in highly complexed systems (Okabe et al., 1999; Gieseke et al., 2003; Schramm et al., 2000) (Table.1).

Table. 1. Fluorescent oligonucleotide probes used for the detection of nitrifiers by various researchers

Ammonia oxidizers			
Ammonia oxidizing ß-Proteobacteria -			
Nso 1225	Ammonia oxidizing ß-Proteobacteria -	CGCCATTGTATTACGTGTGA	Mobarry et al., 1996
NSO190	Ammonia-oxidizing ß Subclass *proteobacteria*	CGATCCCCTGCTTTTCTCC	Mobarry et al., 1996
ß-AO233	All ß-subgroup ammonia oxidizers	AGCTAATCAGRCATCGG	McCaig et al., 1999
NITROSO4E	ß proteobacterial AOB	CACTCTAGCYTTGTAGTTTC	Burrell et al., 2001
AAO-258	Non-marine ß ammonia oxidizers	GGTAAAGGCTTACCAAGGC	
Nitrosomonas			
Nmo254	*All Nitrosomonas* spp.	GTAGGCCSTTACCCYACC	McCaig et al., 1999
Nm75	*Nitrosomonas genus*	CGGCAGCGGGGGCTTCGGCC	
Nm0 (148-165)	*Nitrosomonas genus*	ATAACGCATCGAAAGATG	
NSM156	*Nitrosomonas* spp.	TATTAGCACATCTTTCGAT	Mobarry et al., 1996
NEU	Halophilic and halotolerant members of the genus *Nitrosomonas*	CCCCTCTGCTGCACTCTA	Wagner et al., 1995
NSMR76	*Nitrosomonas marina* like *AOB*	CCCCCCTCTTCTGGATAC	Burrell et al., 2001
NmoCL6b_376	*Nitrosomonas* cluster 6b	GGATCAGGCTTGCGCCC	McCaig et al., 1999
NmoCL7_439	*Nitrosomonas* cluster 7	CTCTTTCTTTCCGACTAA	McCaig et al., 1999
NSM1B	*Nitrosomonas europaea, Nitrosomonas eutropha, NItrosoccocus mobilis*	TCTGTCGGTACCGTCAT	Hovanec and Delong, 1996
NmII	*Nitrosomonas communis* lineage	TTAAGACACGTTCCGATGTA	Pommerening –Roser et al., 1996.
NmIV	*Nitrosomonas cryotolerans* lineage	TCTCACCTCTCTCAGCGAGCT	Pommerening Roser et al., 1996.
Nitrosospira			
Nsp436	All *Nitrosospira*	TTTCGTTCCGGCTGAAAG	
NSV443	*Nitrosospira* spp.	CCGTGACCGTTTCGTTCCG	Mobarry et al., 1996
NspCL1_249	*Nitrosospira* cluster 1	CTTTTACCTTACCAACAA	McCaig et al., 1999
NspCL2_458	*Nitrosospira* cluster 2	TCACAGTTATTAACCGTG	Stephen et al., 1998
NspCL3_454	*Nitrosospira* cluster 3	RGGTATTAGCCGTGACCG	Stephen et al., 1998
NspCL4_446	*Nitrosospira* cluster 4	ACCGTAACCTTTTCGTTC	Burrell et al., 2001
NSMR34	*Nitrosospira tenuis* like AOB	TCCCCCACTCGAAGATACG	

Nitrosococcus			
Nm V	*Nitrosococcus mobilis* lineage	TCCTCAGAGACTACGCGG	Juretschko et al., 1998
	Nitrite Oxidizers		
Nitrobacter			
NIT2	*Nitrobacter* species	CGGGTTAGCGCACCGCCT	Wagner et al., 1996
NIT3	*Nitrobacter* spp	CCTGTGCTCCATGCTCCG	Wagner et al., 1996
NBAC2	*Nitrobacter winogradskyi* *Nitrobacter hamburgensis* *Nitrobacter agilis*	GCTCCGAAGAGAAGGTCACA	Hovanec and Delong, 1996
Nitrospira			
Ntspa712	Phylum *Nitrospira*	CGCCTTCGCCACCGGCCTTCC	
Ntspa662	Genus *Nitrospira*	GGAATTCCGCGCTCCTCT	
NSR826	Freshwater *Nitrospira* spp	GTAACCCGCCGACACTTA	Schramm et al., 1998
NSR 1156	Freshwater *Nitrospira* spp	CCCGTTCTCCTGGGCAGT	Schramm et al, 1998
S-G-Ntspa-0685-a-A-22	*Nitrospira* like AOB	CACCGGGAATTCCGCGCTCC TC	Burrell et al., 2001
Ntspa685	*Nitrospira moscoviensis*, *Nitrospira marina*, aquarium clone 710-9	CACCGGGAATTCCGCGCTCC TC	Hovanec et al., 1998
Ntspa454	*Nitrospira moscoviensis*, aquarium clone 710-9	TCCATCTTCCCTCCCGAAAA	Hovanec et al., 1998
Ntspa1026	*Nitrospira moscoveinsis*, activated sludge clones A-4 and A-11	AGCACGCTGGTATTGCTA	Juretschko et al., 1998
	Anaerobic ammonium-oxidizers		
S-*-Amx-0820-a-A-22	Anaerobic ammonium-oxidizing bacteria	AAAACCCCTCTACTTAGTGCCC	Schmid etal., 2003

Denaturing gradient gel electrophoresis (DGGE) of PCR amplified genes another recently developed and widely used technique for evaluating the diversity of nitrifiers in complex microbial systems (Kowalchuk et al., 1997; Stephen et al., 1998; Burrel et al., 2001; Tal et al., 2003). In DGGE, the separation of equal length DNA fragments is based on sequence-dependent melting behavior in a polyacrylamide gel containing a concentration gradient of increasing denaturant (Muyzer et al., 1998). Studies of AOB diversity using DGGE have until recently been restricted to 16S rRNA gene analysis but now also make use of the functional amoA genes (Nicolaisen and Ramsing, 2002).

Molecular characterization of nitrifying bacterial commununities will be incomplete when neglecting the anaerobic ammonia oxidizers (Annamox) and the archaeal ammonia oxidizers. Like for autotrophic nitrifiers, several probes and primers are available for the detection of these organisms. Schmid et al. (2005) reviewed all the available biomarkers for the detection of annamox bacteria. Archaeal specific primers (Delong, 1992) and archaeal amoA primers (Fransis et al., 2005) are now widely used for the detection of archaeal ammonia oxidizers.

Recent studies on "nitrifier denitrification" further complicate the separation of denitrification from the nitrification process (Cantera and Stein, 2007). Some ammonia-oxidizing *Nitrosomonas* spp. are capable of anaerobic growth on hydrogen by reducing NO_2^- to N_2 via the activities of nitrite and nitric oxide reductases (Bock, 1995; Schmidt et al., 2004). In addition, both *Nitrosomonas* and *Nitrosospira* spp. reduce NO_2^- and release substantial quantities of the potent greenhouse gas nitrous oxide (N_2O) under aerobic to suboxic conditions (Dundee and Hopkins, 2001; Shaw et al., 2005). Similarly, the nitrite-oxidizing *Nitrobacter* spp. produces N_2O during anaerobic respiration of NO_3^- and NO_2^- (Bock et al., 1988). The reduction of NO_2^- to NO, an intermediate step in denitrification, is catalysed by nitrite reductase. Two main classes of dissimilatory nitrite reductases exist among denitrifying bacteria: the haemcytochrome *cd*1 type encoded by *nirS* genes, and the copper-containing type encoded by *nirK* genes (Hochstein and Tomlinson, 1988; Glockner et al., 1993; Zumft, 1997). Various primers are now available for the amplification of these nitrite reductase genes, nirK and nirS (Braker et al., 1998; Hallin et al., 1999).

In the study undertaken here four nitrifying bacterial consortia developed by enrichment technique (Achuthan et al., 2006) and used for activating the bio reactors for aquaculture systems were subjected for characterization. The ammonia and nitrite oxidizing consortia for penaeid systems were developed at 30 ppt (AMOPCU-1-Ammonia oxidizing consortia for penaeid culture and NIOPCU-1- Nitrite oxidizing consortia for penaeid culture) and those for non-penaeid systems were developed at

15 ppt (AMONPCU-1 - Ammonia oxidizing consortia for penaeid culture and NIONPCU -Nitrite oxidizing consortia for non-penaeid culture). For the characterization of these consortia of different salinities, a polyphasic approach was followed using various tools such as electron microscopy, amplification and random cloning of functional as well as 16S rRNA gene, Florescence in situ hybridization (FISH) and amplified ribosomal DNA restriction analysis (ARDRA) for the community analysis.

2.2 Materials and methods

2.2.1 Ultrastructure of the consortia

Transmission electron microscopy (TEM) of the four consortia was carried out by the paraformaldehyde-glutaraldehyde-ruthenium red-lysine extended time following Fassel and Edmiston Jr (1999). The cultures were centrifuged (4000 × g, 10 min at 4°C) and the pellets completely resuspended in the primary fixative solution. During each further stage of the processing before dehydration, the cells were centrifuged (12000×g, 2 min at 4°C) and the pellets were suspended carefully in the next reagent. The primary fixative contained 75mM lysine in buffered 0.075% (w/v) ruthenium red, 2% (w/v) paraformaldehyde, 2.5% (w/v) glutaraldehyde and fixed for extended time of 24 hr. Pellets were washed three times for 10 min in 0.1 M sodium cacodylate buffer (pH 7.2). Post fixation was in 1% (w/v) OsO_4, 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hr. Resulting pellets were washed three times for 10 minutes, in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated. The buffers were prepared in filtered autoclaved seawater according to the salinity optima of the consortia. The dehydration was carried out through an acetone series of 70-100 %. After dehydration cells were embedded in epoxy resin, sectioned and stained with lead citrate and uranyl acetate and examined under the electron microscope (Morgagni 268-D, Netherlands).

2.2.2 Fluorescence *in situ* hybridization (FISH)

Fluorescence in situ hybridization analysis of the four consortia was carried out using eight different 16S rRNA targeted oligonnucleotide probes labeled with

either Cy3, Cy5 or Fluorescein (Table 2). The fluorescent oligonucleotide probes were purchased from Thermo Electron Corp. (Germany). The specificity and the hybridization conditions were confirmed with 'Probebase' (Loy et al., 2007). Actively growing consortia were harvested by centrifugation were fixed in 4% paraformaldehyde in PBS (prepared with respective salinity). The samples were stored at -20°C in a 1:1 mixture of PBS:ethanol until further processing.

2.2.2.1 Pretreatment of the microscopic slides

Hybridizations were performed on 6- well Teflon-coated slides (Electron Microscopy Sciences, USA). Prior to the hybridization, the slides were coated with poly L-Lysin wherein the slides were first cleaned with 70% ethanol and then placed in the 100 ml coplin jars containing 0.01% poly L-Lysin solution for 5 min. After that the slides were dried overnight at room temperature in a vertical position

2.2.2.2 Immobilization of cell on microscope slides

10µL of each fixed consortia were spread in the well of the slide, dried at 46°C for 10 min, and then dehydrated by successive passage through 50, 80 and 98% ethanol (3 min each). After that the slides were allowed to dry at room temperature for 4 min.

2.2.2.3 Hybridization

Working solutions of the probes were prepared to obtain a final concentration 5 pmol/µL for CY3/5 and 8.3 pmol/µL for fluorescein labeled probes. Hybridization buffer (2 ml) containing 360µL 5M NaCl, 40 µL 1M Tris-HCl (pH 8.0), 4µL 10% SDS, formamide and MilliQ was prepared and taken according to the probe used (Table.2). For hybridization 10µL of hybridization buffer was dispensed into the wells, and then 1 µL of probe stock solution was added without scratching the Teflon coating. A hybridization tube was prepared by folding a tissue paper into a 50 ml falcon tube into which the remainder of the hybridization buffer was dispensed. After the addition of probes the slides were immediately transferred into the hybridization tube and incubated for 1.5 hr at 46°C in a hybridization oven

(Thermo Electron Corp). Washing buffer (50 ml) containing 1M Tris/HCl, 5M NaCl and 0.5M EDTA, pH 8 was prepared as per the formamide concentration in the hybridization buffer in a separate 50 ml Falcon tube and made up to 50ml by adding MilliQ. Finally 50µL of 10% (w/v) SDS was added and the washing buffer was preheated at 48°C in a water bath. On elapse of the incubation period, the hybridization slides were taken out and rinsed with the washing buffer and transferred into the washing buffer. Inside the washing buffer the slides were incubated for 10-20 min at 48°C. After the incubation washing buffer was removed by rinsing with MilliQ water and the slides were dried. The cells were then counter-stained with DAPI (4,6- diaminidino-2-phenylindole; final concentration of 0.2µg/ml for 1 min, washed again and dried. Before observing in an epi-fluorescent microscope, an ant-fading mounting fluid (Vectashield, Vector laboratories Inc., Burlingame, CA) was added. The slides were observed under Olympus BX 51 epifluorescent microscope equipped with a monochromatic camera (Evolution VF, Media Cybernetics Inc, MD, USA). Images were processed using the "Image pro-express" software (Media cybernetics Inc, MD, USA).

2.2.3 Molecular characterization

2.2.3.1 DNA Extraction

DNA from the four consortia was extracted following Burrel et al. (1998). Two millilitres of the consortia was centrifuged at 12000 × g for 5 min. The supernatant was discarded and the pellet resuspended in 500 µL of saline EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0). A volume of 100 µL of freshly prepared 100 mg/ml lysozyme was added to the mixture and incubated at 37°C for 1.5 hr. The mixture was then subjected to four cycles of freeze-thaw sequentially at -20 and 65°C. Thereafter 100 µL of 25 % (w/v) sodium dodecyl sulfate and 50 µL of 2 % (w/v) proteinase K were added to the mixture and the mixture was incubated at 60°C for 1.5 hr. The solution was deproteinated by the addition of phenol-chloroform-isoamyl alcohol mixture (25:24:1). Total nucleic acids from the 0.5 ml aqueous phase was precipitated by incubating for 1 h at -70°C after adding 0.1 volume of sterile 3 M sodium acetate and 1 vol of ice-cold 100 % ethanol. The DNA pellet

was recovered by centrifuging the solution at 12000 × g for 20 min at 4°C. Salts and other impurities were removed by washing the pellet in 500 µL of 70 % ice-cold ethanol and DNA was recovered by centrifugation at 12000 × g for 10 min at 4°C. The pellet was then air dried, and the nucleic acids were dissolved in 100 µL of sterile deionized water (MilliQ). Residual RNA was removed from the nucleic acid solution by adding 3 µL of 10 mg/ml RNAse and incubating at 37°C for 1 hr. The DNA was visualized by over 0.7% agarose gels and concentration was assessed spectrophotometrically (A_{260}/A_{280} nm). Working stocks of the DNA were prepared at a concentration of 100 ng and maintined at -20°C until further use.

2.2.3.2 PCR amplification of the phylogenic and functional genes

Phylogenetic analysis was carried out employing universal bacterial 16S rRNA PCR primers, 16S rRNA gene primers for the specific amplification of 16S rRNA gene fragment of the β- proteobacterial ammonia oxidizers, archaeal rRNA gene fragment and the specific primers for functional genes such as ammonia monoxygenase (*amo* A) specific for chemolithoautotrophic ammonia oxidizers, and the nitrite reductase (nir) genes specific for the denitrifiers.

2.2.3.3 Amplification of the universal bacterial 16S rRNA gene fragment

A 1500 bp bacterial 16S rRNA gene fragment was amplified using the universal primers. A total of three primers (Reddy et al., 2000; Gauzer and Gomez Chiarri, 2002; Humphry et al., 2003) were screened for the amplification of bacterial 16S rRNA gene fragment from the four consortia. After observing the amplicons in the 1% agarose gels, the primer 1 (Reddy et al., 2000) which gave sharp band without non-specific amplification was selected for further reactions.

2.2.3.4 Amplified ribosomal DNA restriction analysis (ARDRA)

A 1500 bp I6S rRNA bacterial gene fragment from the four consortia was amplified as described above. From the 25µL PCR product 10 µL were loaded for observing the presence on an agarose gel while the remainder (15 µL) was used for restriction analysis. The restriction enzymes with maximum loci in the amplified 16S rRNA gene were selected from a pool of 8 randomly chosen

enzymes namely, *Eco*R I, *Bam*H I, *Hin*D III, *Dra* I, *Kpn* I, *Pst* I , *Sal* I and *Xba* I (New England Biolabs). The digestion mix consisted of 15 µL amplified product, 0.5 µL enzyme, 2 µL buffer (supplied with each enzyme), 0.2 µL BSA (wherever required). The digestion mixture was incubated at 37°C for 3 hr. The digests were separated over 2% agarose gel in TAE buffer. Based on the digestions obtained *Bam*H I and *Dra* I enzymes were selected for further analysis. To increase the number loci, double digests of the amplified products from the consortia were carried out using the enzymes *Bam*H I and *Dra* I as per manufacturer's instructions. In a total reaction volume of 20µL containing 0.5µL of each enzymes, 2 µL buffer, 0.2µL BSA and 15 µL amplified product, digestions was carried out overnight at 37°C. The digested products were separated over 15% SDS-PAGE and the gel silver stained for visualizing the resulting bands. The band patterns were scored for the absence/ presence (0/1) of individual loci and analysed using the program NTSYSpc (version 2.02i, Applied Biostatistics). The data matrix prepared in MS-Excel spreadsheets was converted to proprietory matrix files by the programme NT edit (version 1.1b, Applied Biostatistics Inc) and rectangular data matrix generated was analysed by the programme NTSYSpc. Similarities were calculated by the simple matching coefficient using statistical module, sequential agglomerative hierarchial nested cluster method (SAHN) and clustering was achieved by unweighted pair group method with arithmetic mean (UPGMA).

2.2.3.5 Amplification of 16S rRNA primer specific for β-ammonia oxidizing bacteria

The primers NitA (5' CTT AAG TGG GGÃ ATA ACG CAT CG 3') and NitB (5' TTA CGT GTG AAG CCC TAC CCA 3') (Voytek and Ward, 1995) were used for the detection of β- proteobacterial ammonia oxidizers from the consortia. They amplify a 1080 bp fragment near the 5' end of the rRNA genes. Amplifications were performed using PCR thermal cycler (Eppendorf). Reactions were carried out in 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 20mM Tris HCl, pH 8.8), 200 µM each dNTPs, 1 µM of each NitA and NitB, 1 µL template DNA (100-200ng), and 2 U of Taq DNA

polymerase (New England Biolabs) in a total volume of 100 µL. The amplification reactions were carried out using the following cycle: Initial denaturation of the template at 95°C for 3 min, prior to the addition of the Taq DNA polymerase; 82°C for 2.5 min, 54°C for 1.5 min and 72°C for 2.5 min; followed by 35 cycles of 94°C for 1 min, 54°C for 1.5 min and 72°C for 2.5 min followed by a 10 min final extension at 72°C.

2.2.3.6 Amplification of archaeal rRNA gene fragment

The primers Arch21F (5' TTC CGG TTG ATC CYG CCG GA 3'), Arch 958R (5' YCC GGC GTT GAM TCC AAT T 3') (Delong, 1992) yielding an amplicon of 950 bp were used for analyzing the presence of Archaea in the consortia. A volume of 100µL reaction mix contained 1X PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X100, 20 mM Tris-HCl, pH 8.8), 200µM each dNTPs, 0.2 µM each primers, template DNA 1 ng/µL, 2.5 U of Taq polymerase. Thermal cycling was as follows: denaturation at 95°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 1.5 min for a total of 30 cycles.

The amplicons obtained from the above amplifications were observed in 1 % agarose gel and the size of the product was determined by comparing with molecular weight marker (1kb ladder, New England Biolabs) and documented the results using the Gel documentation system (DolphinDoc, Wealtec)

2.2.3.7 Amplification of the functional genes

2.2.3.7.1 PCR amplification of *amo*A

For the amplification of the amoA gene *amo*A-1F (5'GGGGTTTCTACTGGTGGT 3') *amo*A 1R (5' CCCCTCKGSAA AGCCTTCTT C 3') (K= G or T; S=G or C) primers system (Rotthauwe et al., 1997) were used. The primers generate a 491 bp PCR product. The reactions were performed in a solution containing 1X PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X100, 20 mM Tris-HCl, pH 8.8) 20 nmol of each dNTPs, 30 pmol of each primer, 1 µL of template DNA (100ng) and 2.5 U of Taq DNA polymerase (New England Biolabs). The enzyme was added after

the first denaturation step. The standard thermal profile used for the amplification of the amoA target sequence was as follows: 5 min at 94°C; pause at 80°C to add polymerase; then 42 cycles consisting of 90 s at 56.8°C (annealing), 90 s at 72°C (elongation), and 60 s at 94°C (denaturation); and a final cycle consisting of 90 s at 60°C and 10 min at 72°C. Aliquots (10 µL) of the PCR products were electrophoresed and visualized in 1 % agarose gels by using standard electrophoresis procedures.

2.2.3.7.2 Amplification of the nitrite reductase genes (*nir*K and *nir*S)

For the amplification of nirK and nirS genes, *nir*K1F (5'GG(A/C) ATG GT(G/T) CC(C/G) TGG CA3'), nirk5R (5'CTC GAT CAG (A/G)TT (A/G)TG G3') and nirS1F (5'CCT A(C/T)T GGC CGC C(A/G)C A(A/G)T3'), *nir*S6R (5'CGT TGA ACT T(A/G)C CGG T3') primers were used (Braker et al., 1998). PCR amplifications were performed in a total volume of 25 µL containing 1X PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X100, 20 mM Tris HCl, pH 8.8), 500 µM each dNTPs, 2.5 µL of each primer (10 pmol/µL), and 1 µL of template DNA (100 ng). After an initial denaturation at 94°C for 5 min, 1 U Taq polymerase (New England Biolabs) was added and a touch down PCR performed on a thermal cycler (Mastercycler, Eppendorf). The first 10 cycles consisted of a denaturation step at 94°C for 30 s, primer annealing step at 45°C to 40°C (0.5°C decreased by every cycles) for 40 s and elongation was performed at 72°C for 1 min. Thereafter 30 cycles were performed with annealing at 43°C for 40 s and final incubation at 72°C for 10 min was performed. The amplification products (10µL) were analysed by electroporesis on 1% (w/v) agarose gels. Expected amplicon size for nirS1F- nirS6R and nirK1F-nirK5R were 890 and 512 bp respectively (Braker et al., 1998).

2.2.3.2 Cloning of universal 16S rRNA and *amo*A genes

Fresh PCR products of 16SrRNA and *amo*A genes were used for cloning into the pGEM-T Easy vector (Promega). The ligation mix (10 µl) consists of 5 µL ligation buffer (2X), 0.5 µL the vector (50 ng/µL), 3 µL of PCR product and 1 µL of T4 DNA ligase (3 U/µL). The ligation mix was incubated at 4°C overnight. The

ligated mix entirely was used to transform *Escherichia coli* JM109 competent cells prepared using calcium chloride method. The ligation mix was briefly centrifuged and added to 10 ml glass tube previously placed in ice to which 50 µL of competent cells were added and incubated on ice for 20 min. A heat shock at 42°C was given for 90 s, immediately the tubes were placed on ice for 2 min and then 600µL of SOC media was added and incubated for 2 hr at 37°C with shaking at 250 rpm. The transformation mixture (100-200 µL) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM) and X-gal (80 µg/ml). The plates were incubated at 37°C overnight. The clones were selected using the blue/white screening. The white colonies (100 nos.) were selected and streaked to purity on LB-Amp+-X-gal+-IPTG+ plates and incubated at 37°C overnight. To confirm the insert colony PCR of the white colonies were carried out using the vector primers (T7(5' TAATACGAC TCACTATAGGG) and SP6 (5' GATTTAGGTGACACTATAG) White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25µL) containing 2.5µL 10x PCR buffer, 2.5µL of 2.5mM dNTPs, 1µL of 10 pmol/µL of T7 and SP6 primers, 0.5 U Taq polymerase, and the remaining volume was made up with MilliQ. The thermal cycling conditions were as follows: 1 × 95°C for 5 min; 35 × (94°C for 15 s, 57°C for 20 s, 72°C for 60 s); 1 × 72°C for 10 min following which the temperature was brought down to 4°C. Plasmids from the positive clones were extracted using the 'GenElute HP' plasmid miniprep kit (Sigma). An overnight recombinant *E. coli* culture was harvested with centrifugation and subjected to a modified alkaline-SDS lysis procedure followed by adsorption of the plasmid DNA onto silica (column) in the presence of high salts. Contaminants were removed by spin wash step. Finally, the bound plasmid DNA is eluted in 5 mM Tris-HCl pH 8.0. Plasmids were further screened by restriction digestion using *Eco*R1 enzyme to release the insert. The reaction mix (20 µL) consists of 2 µL of 10X buffer (NEB- *Eco*R1 buffer), 5 µL of plasmid DNA , 0.5 µL of *Eco*R1 enzyme (20,000 U/ml) and the rest made up using sterile water (Milli Q). The reaction mix was incubated at 37°C for 1 hr and the enzyme was heat inactivated at 65°C for 20 min. Positive clones were

cryopreserved under 10% glycerol at -80°C in LB-Amp+ media for further analysis.

2.2.3.3 Sequencing of the clones and sequence analysis

The purified plasmids were sequenced using ABI Prism 3700 Big Dye sequencer at Microsynth AG, Switzerland. The primers used for sequencing were T7 and amoA-1F. The sequences obtained were first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). After removing contaminating vector regions, the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al 1990) also from the NCBI website. The sequences were multiple aligned using the program ClustalW (Thompson et al., 1994). A neighbourhood-joining (NJ) consensus tree was generated based on UPGMA with the Kimura two-pramater model (Kimura, 1983) using the software DAMBE ver 4.5 (Xia and Xie, 2001) where the bootstrap values were fixed at 100. Comparisons were made amongst individual clones and between clones and the sequences to which they showed maximum homology during the BLAST match.

2.3 Results and discussions

2.3.1 Ultrastructure of the consortia

Preliminary characterization using transmission electron microscopy of the consortia demonstrated the presence of characteristic cyst formation and the presence of cytomembranes resembling the autotrophic nitrifiers (Fig.1). Carboxysomes and/or polyphosphate like inclusions were observed in some cells. A striking feature was the embedded nature of majority of cells in an outer glycocalyx. In nature and in enrichment cultures, many of the nitrifying bacteria occur in cell aggregates referred to as zoogloea or cysts. Zoogloea is composed of loosely associated cells embedded in a soft slime layer, cysts are comprised of closely packed cells embedded and surrounded by a firm slime layer. Some of the nitrifying bacteria possess intracytoplasmic membranes which may occur as flattened lamellae or randomly arranged tubes. For example, in *Nitrobacter* sps

the cytoplasmic membrane infolds into the cytoplasm, forming a polar cap of intracytoplasmic membranes composed of 4-6 layers of paired membranes (Watson et al., 1989). So far very few studies were done on the electronmicroscopic analysis of intact communities of nitrifying bacteria. Puzyr et al. (2001) studied the colony produced by a consortium of nitrifying bacteria using light and electron microscopy and they described the morphological features of colonial communities in sufficient detail for their preliminary identification. Based on these studies the ultra structure of the four nitrifying consortia could be demonstrated to match with the previously described nitrifiers

2.3.2 Fluorescence *in situ* hybridization (FISH) of the consortia

Fluorescence in situ hybridization analysis of the four consortia with one general bacterial probe and other seven nitrifying bacterial specific probes confirmed the presence and diversity of the autotrophic nitrifiers in the consortia. Taking the consortia altogether except the probes NEU (Halophilic and halotolerant members of the genus *Nitrosomonas*) and S-Amx-0820-a-A-22 (Anaerobic ammonium oxidizing bacteria) all others gave positive signals. This showed the consortia having mainly consisting of aerobic autotrophic nitrifiers. Most of the nitrifiers observed in the consortia were in the form of aggregates. FISH analysis of the 15ppt ammonia oxidizers (Fig. 2) detected the presence of β ammonia oxidizers and the ammonia oxidizers coming under the *Nitrosococcus mobilis* lineage of β ammonia oxidizers. Long actinomycetes like cells were observed in the DAPI staining, but the bacterial probe EUB 338 was unable to detect these forms. The presence of *Nitrobacter* sp. was also observed. FISH observations of the 30 ppt ammonia oxidizers (Fig.3) also showed the presence of the β ammonia oxidizer, *Nitrosospira* in place of *Nitrosococcus mobilis* lineage observed in the 15 ppt ammonia oxidizers. FISH analysis of the 15 ppt (Fig. 4) and 30 ppt (Fig.5) nitrite oxidizing consortia observed the presence of *Nitrobacter* sps. in the 15ppt and the *Nitrospira* in the 30 ppt nitrite oxidizers.15 ppt nitrite oxidizers also carry the ammonia oxidizers belonging the sub class β-proteobacteria. But the 30ppt nitrite oxidizing consortia were devoid of any ammonia oxidizers targeted by the

selected probes. DAPI staining of the cells also showed a least morphological diversity in the 30 ppt nitrite oxidizing consortia. No anaerobic ammonia oxidizer was detected from the consortia using the probe S-Amx-0820-a-A-22.

FISH is highly effective for detecting specific bacteria and analyzing complex microbial communities, due to the possibility of detecting specific bacterial cells by insitu hybridization using 16S rRNA targeted oligonucleotide probes labeled with a fluorescent compound. To date, FISH using oligonucleotide probes, targeting signature regions of the 16S rRNA of ammonia and nitrite oxidizing bacteria has been successfully applied for phylogenetical identification and quantification in environmental and engineered systems (Juetschko et al., 1998; Mobarry et al., 1996; Okabe et al., 1999, Schramm et al., 1996; Schramm et al., 1998; Schramm et al., 1999; Wagner et al., 1998, Wagner et al., 1995). Nitrifying bacteria were found to exhibit various organizational forms under different conditions of substrate composition and concentration. Ammonia-oxidizing bacteria were dominant in ammonia-rich inorganic wastewater, while heterotrophic bacteria and ammonia oxidizing bacteria were localized at different positions in the biofilm in organic wastewater. The dynamics of the microbial ecology in the biofilm with regard to the spatial distribution of ammonia-oxidizing bacteria and heterotrophic bacteria caused by gradual change in substrate composition was successfully monitored by FISH analysis (Aoi et al., 2000). Rowan et al. (2003) studied the composition and diversity of ammonia-oxidizing bacterial communities in a biological aerated filter (BAF) and a trickling filter. A greater diversity of AOB was detected in the trickling filters than in the BAF though all samples analyzed appeared to be dominated by AOB most closely related to *Nitrosococcus mobilis*.

Natural, low ammonia environments commonly harbour *Nitrosospira* spp (Hiorns et al., 1995; Kowalchuk et al., 2000) where as engineered high ammonia environments often exhibit a predominance of *Nitrosomonas* spp. (Juretschko et al., 1998, Wagner et al., 1995, Mobarry et al., 1996). Several studies have

suggested that environmental factors such as salinity and ammonia concentrations select for certain species of AOB (Pommerening-Roser et al., 1996, Kowalchuk and Stephen, 2001, Koops and Pommerening-Roser, 2001). In wastewater treatment systems (full-and labscale) there appears to be selection for either predominance of single AOB populations (eg. *N. europaea*-like organisms (Okabe et al., 1999; Schramm et al., 1996); *N. mobilis* like bacteria (Juretschko et al., 1998); *Nitrosospira* like bacteria (Schramm et al., 1998) or several different AOB populations occur together (Abd El Haleem et al., 2000; Gieseke et al., 2001; Daims et al., 2001). It has recently been suggested that the level of AOB diversity found in a reactor relates to the stability of the reactor (Daims et al., 2001). Hence engineering a system with a greater diversity may improve performance and stability. Considering the above, the nitrifying consortia studied here are having diverse nitrifiers, well suited for the activation of the nitrifying bioreactors used in recirculating aquaculture systems.

2.3.3 Molecular characterization of the consortia

2.3.3.1 DNA extraction

Good quantity (300-400ng/μL) and quality (260/280 ratio between 1.75-1.8) of genomic DNA was obtained (Fig.6) from the consortia by following the method used. DNA extraction from consortia requires treatment to disrupt bacterial cells regardless of their biochemical composition probably because of the cells being embedded in a dense glycocalyx. The efficiency of the extraction determines the quantity, quality, and diversity of the extracted DNA or RNA. The cell lysis treatment using lysozyme and SDS, heat shock steps along with phenol-chloroform extraction makes this as a suitable extraction procedure for the nitrifying bacterial consortia inhabited by both Gram-positive and Gram-negative forms.

2.3.3.2 PCR amplification of the phylogenic and functional genes

2.3.3.2.1 Amplification of the universal bacterial 16S rRNA gene fragment

The universal primer set of Reddy et al. (2000) yielded a consistent and specific 1500 bp amplicon and was therefore considered suitable (Fig.7). The primers of Gauger and Gomez-Chiarri (2002) yielded the expected amplicon of 800 bp in all

the consortia except in 30-ppt nitrite oxidizer (NIOPCU). There were however non-specific amplifications with this primer. The primer of Humphry et al. (2003) did not yield any amplicons.

2.3.3.2.2 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Analyzing the restriction pattern of 16S rRNA gene on 2% agarose gel (Fig. 8) the enzymes *Bam*H I and *Dra* I were selected for further analysis. The number of loci could be increased by performing a double digests of the samples with *Bam*H I and *Dra* I. The digested products were well separated on 15 % SDS PAGE and visualized by silver staining (Fig.9). The dendrogram generated based on the ARDRA analysis (Fig. 10) points that there is not much variation in the community structure between the four consortia. Among them the 15ppt ammonia and nitrite oxidizers share almost similar communities while there exists some variation in the 30 ppt ammonia oxidizers community. Although ARDRA gives little or no information about the type of microorganisms present in the sample, it can be used for a quick assessment of genotypic changes in the community over time, or to compare communities subject to different environmental conditions. Smit et al., 1997 used ARDRA for detecting the copper-related changes in the AOB community composition.

2.3.3.2.3 PCR amplification of 16S rRNA -β-ammonia oxidizing bacteria; functional gene -*amo*A, nitrite reductase genes (*nir*K and *nir*S) and archaeal rRNA gene fragment

Using the primers Nit A and Nit B, amplification of 16S rRNA gene fragment (1080 bp) specific for the β-ammonia oxidizers was obtained from the two ammonia oxidizing consortia but not from the nitrite oxidizing consortia (Fig. 11). The functional gene of the ammonia oxidizing bacteria, the ammonia monoxygenase (*amo*A) was also amplified from both the ammonia oxidizing consortia with a product size of 491 bp (Fig. 12). However, this amplification was not observed in the nitrite oxidizing consortia. Amongst the nitrite reductase genes, *nir*K and *nir*S, amplicons (890 bp) of *nir*S were obtained with 30 ppt ammonia oxidizers only (Fig.13). No amplification of *nir*K genes was observed in

any of the consortia, however, non-specific amplifications were observed in all reactions. Archeaeal genes were not detected in any of the consortia.

2.3.3.2.4 Cloning and sequencing of universal 16S rRNA and amoA genes

In order to determine the diversity of the autotrophic nitrifiers in the consortia cloning and sequencing of *amo*A gene and 16S rRNA genes were carried out. For the autotrophic ammonia oxidizers the functional gene *amo*A was used for sequencing. Because of the absence of any functional gene of the nitrite oxidizers, 16S rRNA sequencing was used for the identification. Subsequent to amplification and cloning of these genes from the consortia, several levels of screenings were carried out for the conformation of the insert. The initial screening was by colony PCR using T7 and SP6 vector primers which produced a product of 1.7 kb size (Fig.14) for the 16S rRNA gene insert and 0.7 kb for the *amo*A insert (Fig.15). The clones with no insert or with non targeted inserts were discarded after the colony PCR and the rest were taken for plasmid extraction. The plasmids extracted using the GenElute HP Plasmid miniprep kit (Sigma) were checked for quality and quantity in agarose gel (Fig.16) and spectrophotometrically. A quantity of 200-300 ng/μL was obtained with a 260/280 ratio of 1.8-1.86. Before sending the plasmids for sequencing a secondary screening were carried out for further conformation of the insert. The screening involved the restriction digestion of the plasmids with *Eco*R 1, which released the insert from the plasmids. In Fig. 17, lanes 1-5 show the clones carrying the *amo*A gene, which released the *amo*A insert (491bp) and the plasmids were separated (3kb) after the digestion. Lanes 6-29 show the expected clone carrying the 1.5 kb 16s rRNA gene. Separation of the insert from the plasmid was observed in all cases. Lanes 6, 19 and 26 have an internal restriction site for *Eco*R 1 enzyme, so the 1.5kb product appeared as two bands.

Sequences from the clones containing *amo*A insert when matched with the GenBank database showed similarities to the *amo*A gene of *Nitrosomonas* sp., *N. europaea, N. eutropha* and *amo*A gene of many uncultured bacteria. The close

matches, above 98% similarity in the blast results were chosen to construct a phylogenetic tree with the sequences obtained (Fig.18). Using the ClustalW programme the sequences of *amo*A gene obtained from 15 and 30 ammonia oxidizing consortia clones (Fig. 19) and 16S rRNA gene obtained from 15 & 30 nitrite oxidizing consortia clones (Fig. 20) were multiple aligned. The similar colouration shows the sequence similarity between the ammonia oxidizing genes and the nitrite oxidizing consortia clones. On matching the 16S rRNA gene sequences obtained with the clones of 15N cultures with the GenBank database using the BLAST algorithm the majority of the hits were with the sequences of uncultured bacteria and other heterotrophs like *Flavobacterium* sp., *F. mizutaii*, Bacteroidetes *bacterium* and uncultured *Sphingobacterium* sp.,while that of 30N clones blast with *Flexibacter tractuosus*, *Microscilla sericea*, *Alcanivorax dieselolei*, *Alcanivorax* sp., *Uruburuia balearica*, Uncultured *Cytophagales* and *Sphingobacteriales* bacterium. However, on comparing these sequences phylogenetically, significant similarities were lacking between these sequences (Fig. 21 a and b). When a diverse PCR product such as the one here is cloned, the resulting clones could either arise from the amplicons of the same species or different species. Since randomly chosen clones were being sequenced, it was important to analyse whether they were from the same organism or different. This was analysed by constructing a NJ tree of the sequences and it showed that there was high degree of similarity between the sequences obtained from 15- and 30–ppt ammonia oxidizing cultures (Fig. 22) indicating that they might have originated from same or closely related organisms. This factor could be attributed to the small number of clones analysed, as usually such approaches demand sequencing of a much larger number. Similar comparisons made with 16S rRNA gene sequences of 15N and 30N cultures (Fig. 23) also showed similar results. Therefore, it may not be possible to arrive at a conclusion based on this data; nevertheless, prima facie this indicates that these consortia consist of novel organisms hitherto unreported.

The aerobic autotrophic ammonia-oxidizing bacteria (AOB) were found within two phylogenetic groups based on comparison of 16S rRNA gene sequences (Head et al., 1993; Teske et al., 1994; Purkhold et al., 2000). One group comprises strains of *Nitrosococcus oceani* and *Nitrosococcus halophilus* within the class γ-proteobacteria and the other contains *Nitrosomonas* and *Nitrosospira* spp. within the class β-proteobacteria and most of the molecular studies were targeted towards this β-subclass of ammonia oxidizers (Stehr et al., 1995; Voytek and Ward 1995; Pommerening-Roser et al., 1996; Bano and Hollibaugh, 2000). It should be noted that *Nitrosococcus mobilis* is misnomer and in fact belongs to the β-proteobacterial genus *Nitrosomonas* not the genus *Nitrosococcus* in the γ-proteobacteria. A continually expanding database of AOB 16S rRNA gene sequences has led to the description of distinct clusters within the betaproteobacterial AOB from the family 'Nitrosomonadaceae', five within the genus *Nitrosomonas* and five within the genus *Nitrosospira* (Purkhold et al., 2000). The genus *Nitrosomonas* also contains a lineage currently represented only by *Nitrosomonas cryotolerans* –like sequences (Purkhold et al., 2000).

Amplification of *amo*A gene in the consortia confers the presence of classical ammonia oxidizers in them besides their demonstration through structural gene amplification. This approach has been followed by several workers (Holmes et al., 1995; Sinigalliano., 1995; Rothauwe et al., 1997; Mendum et al., 1999). Ammonia oxidizing bacterial diversity survey performed by Purkhold et al. (2000) in 11 nitrifying wastewater treatment samples using the amoA PCR demonstrated that all clones contained *amo* A sequences affiliated to the β-subclass of ammonia oxidizing bacteria. Aoi et al. (2004) used the *amo* A mRNA profiles to study the changes in the ammonia oxidation activity in a complex microbial community. Nicolaisen and Ramsing (2002) carried out *amo* A gene PCR in conjunction with DGGE to investigate the diversity of ammonia oxidizing bacteria in different habitats. Environmental 16S rRNA and *amo* A gene libraries significantly extended our knowledge on the natural diversity of AOB.

The availability of 16S rRNA gene sequences also provided a basis for the development of cultivation-independent methods to investigate the diversity and community composition of these microorganisms in complex environments. PCR–mediated preferential amplification of AOB 16S rRNA gene and subsequent cloning and sequencing have been extensively applied to create phylogenetic inventories of various environments (Whitby et al., 1999; Bano and Hollibaugh, 2000, Kowalchuk et al., 1997, 1998, 2000)

Homologues of *nir*K and *nor*B have been fully sequenced in *N. europaea*, and partial *nir*K homologues have also been identified in several marine nitrifiers that are capable of denitrification. Regardless of the specific functions of *nir*K and *nor*B in ammonia oxidizing bacteria, the presence of such sequences in ammonia oxidizing bacteria and the potential for different uses and controls relative to denitrifying bacteria complicate the interpretations of functional gene diversity in mixed environmental samples. The gene *nir*S has not been identified in nitrifying bacteria so far, but *nir*K has been reported in both nitrifying and denitrifying bacteria (Casciotti and Ward, 2005).

Recent studies shows that the ammonia oxidizing archaea are dominant populations in various environmental samples (Leninger et al., 2006; Nicol and Schleper 2006; Wuchter et al., 2006). Park et al. (2006) first reported the presence of ammonia oxidizing archaea in wastewater treatment bioreactors. But the possibilities are rare for the existence of archaea in a laboratory culture like the nitrifying consortia studied here which has undergone several sub cultures.

The low node values indicate a low similarity between the sequences already available in GenBank which might indicate a significant evolutionary distance, conformation of which would however need more sequence data. In addition, since these sequences arose from a random clone library it becomes difficult to assign their origin to a particular species. However, detection of the *amo*A gene in only the ammonia oxidizer consortia and not in the nitrite oxidizer, clearly confirms that the functional capacity of these are dictated by the organisms they

harbour and that the functional genes approach to resolve will be successful when coupled with more robust techniques such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE).

Cloning approaches to characterize complex bacterial populations have been unable to resolve such populations in addition to being time consuming and expensive (MacFarlane and MacFarlane, 2004). Therefore to shed light on the members constituting these consortia, PCR coupled with fingerprinting methods such as DGGE and TGGE would need to be utilized. Although these methods have been in vogue to characterize complex bacterial communities and have yielded robust results phylogenetic information they provide is still inconclusive (Myers et al., 1985).

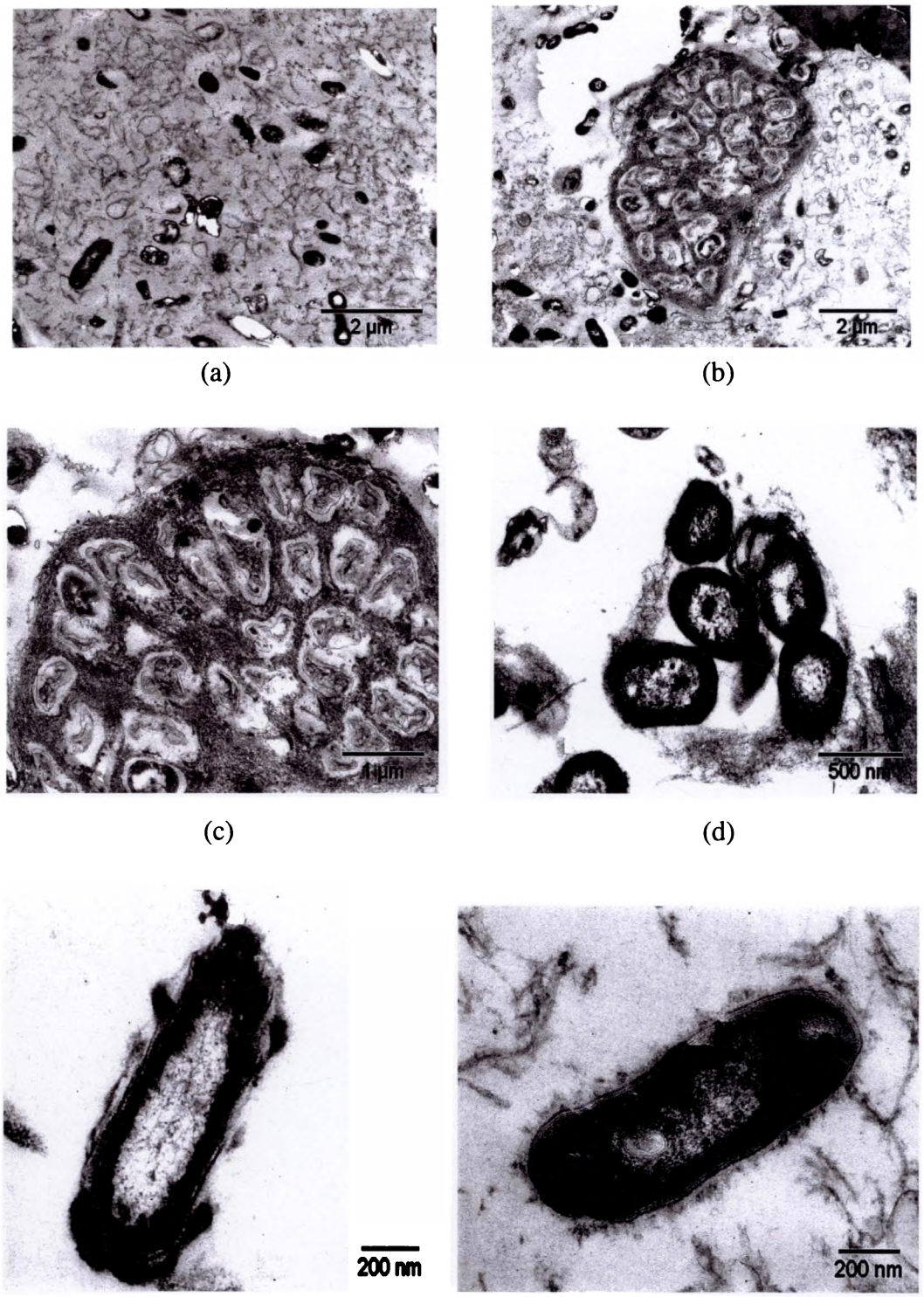
The investigations conducted here to characterize the consortia are with a few limitations. Being consortia composed of several species of organisms together, it is not sure whether DNA has been extracted from all organisms by employing a single method and sufficient representation was there in the reaction mixture while subjected for amplification of 16S rRNA gene and subsequent random cloning. Size of 16S rRNA library constructed was small (80) and the clones sequenced were only 10. With this information the novelty of the organisms noticed can not be fully appreciated. Convincingly, more screening of the library and further sequencing will be required before arriving at the phylogeny of the members in the consortia.

Table. 2. Oligonucleotide probes and hybridization conditions applied in this study

Probe	Target organisms	Probe sequence (5' to 3')	Fluorescent dye used for labeling	FA[%]a	NaCl [mM][b]	Reference
EUB 338	Bacteria	GCTGCCTCCCGTAGGAGT	CY-5	30	112	Amann et al., 1990
NSO190	Ammonia-oxidizing ß Subclass *proteobacteria*	CGATCCCCTGCTTTTCTCC	CY-3	55	20	Mobarry et al., 1996
NEU	Halophilic and halotolerant members of the genus *Nitrosomonas*	CCCCTCTGCTGCACTCTA	CY-5	40	56	Wagner et al., 1995
NSV443	*Nitrosospira spp.*	CCGTGACCGTTTCGTTCCG	CY-3	30	112	Mobarry et al., 1996
Nm V	*Nitrosococcus. mo*bilis lineage	TCCTCAGAGACTACGCGG	Fluorescin	35	80	Pommerening-Roser et al., 1996
NIT2	Nitrobacter species	CGGGTTAGCGCACCGCCT	(Cy-5)	40	56	Wagner et al., 1996.
Ntspa712	Phylum Nitrospira	CGCCTTCGCCACCGGCCTTCC	(CY-3)	50	28	Daims et al., 2001
S--Amx-0820-a-A-22	Anaerobic ammonium-oxidizing bacteria	AAAACCCCTCTACTTAGTGCCC	Fluorescin	40	56	Schmid et al., 2000

a. Formamide concentration in the hybridization buffer

b. Sodium chloride concentration in the washing buffer

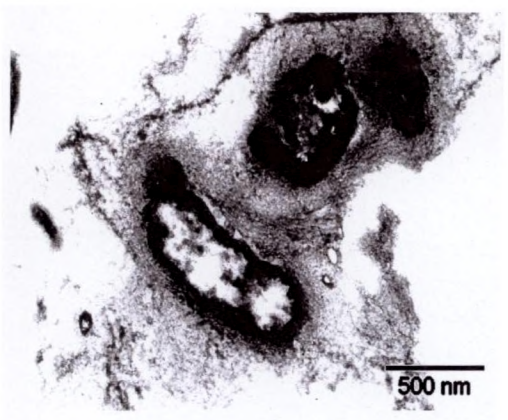


(a) (b)

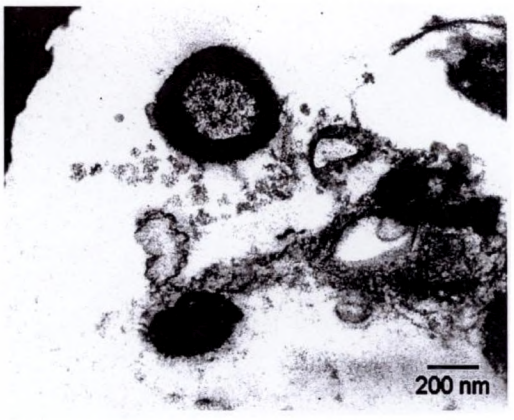
(c) (d)

(e) (f)

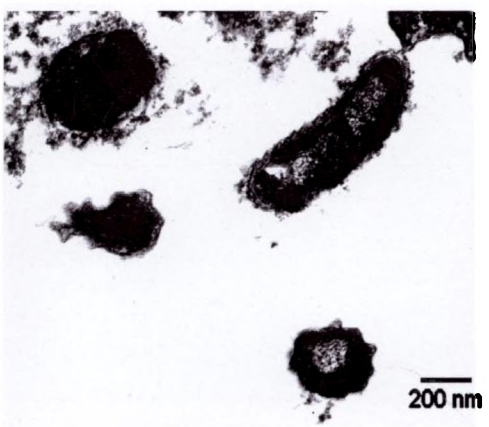
Fig.1. Transmission electron micrographs of the nitrifying bacterial consortia demonstrating glycocalyx (a, f), cyst (b, c), carboxysomes (d, f) and intracytoplasmic membranes (e)



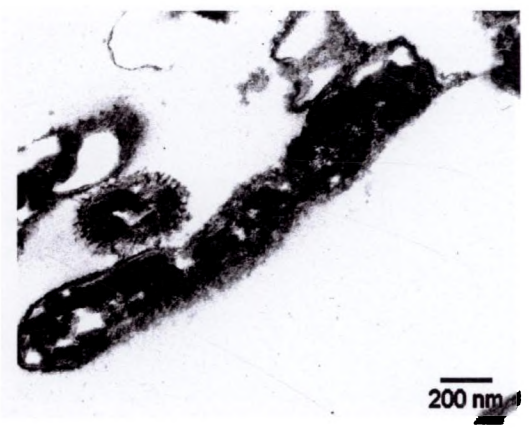
(g)



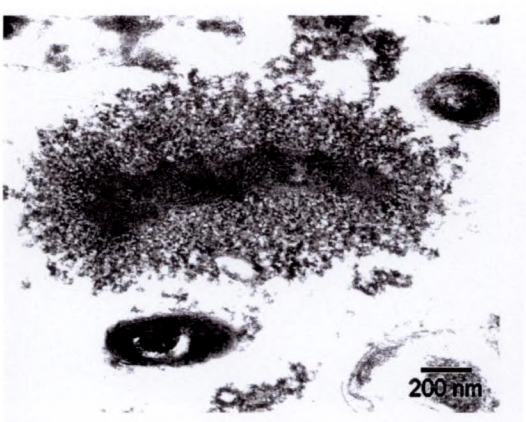
(h)



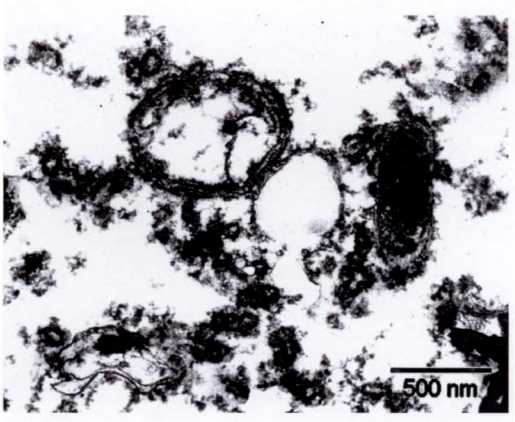
(i)



(j)



(k)



(l)

Fig.1 (Contd.) Transmission electron micrographs of the nitrifying bacterial consortia demonstrating glycocalyx (g, h, i, j, k) and intracytoplasmic membranes (I, j, l)

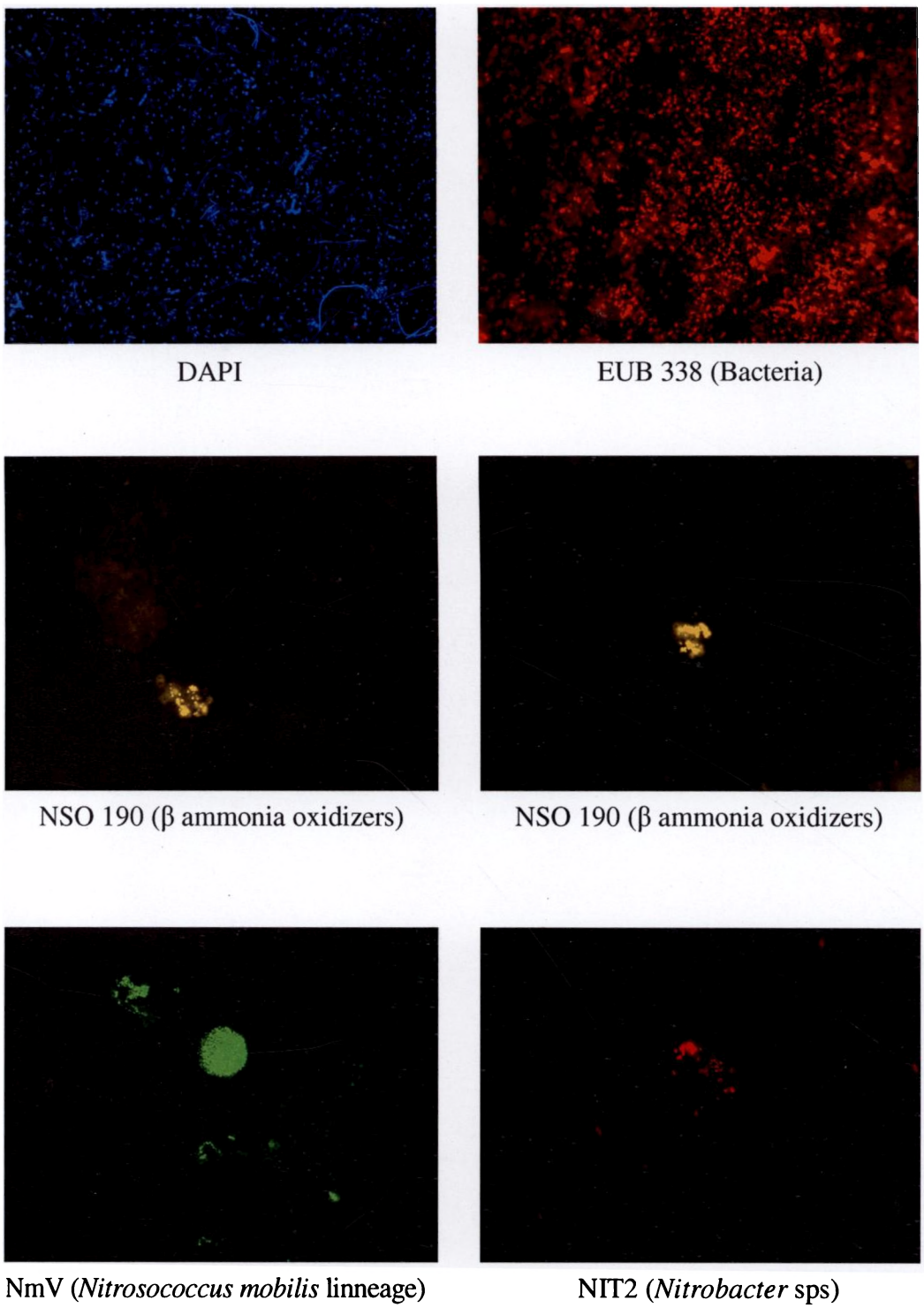


Fig.2. Fluorescence insitu hybridization of the 15 ppt ammonia oxidizing consortia

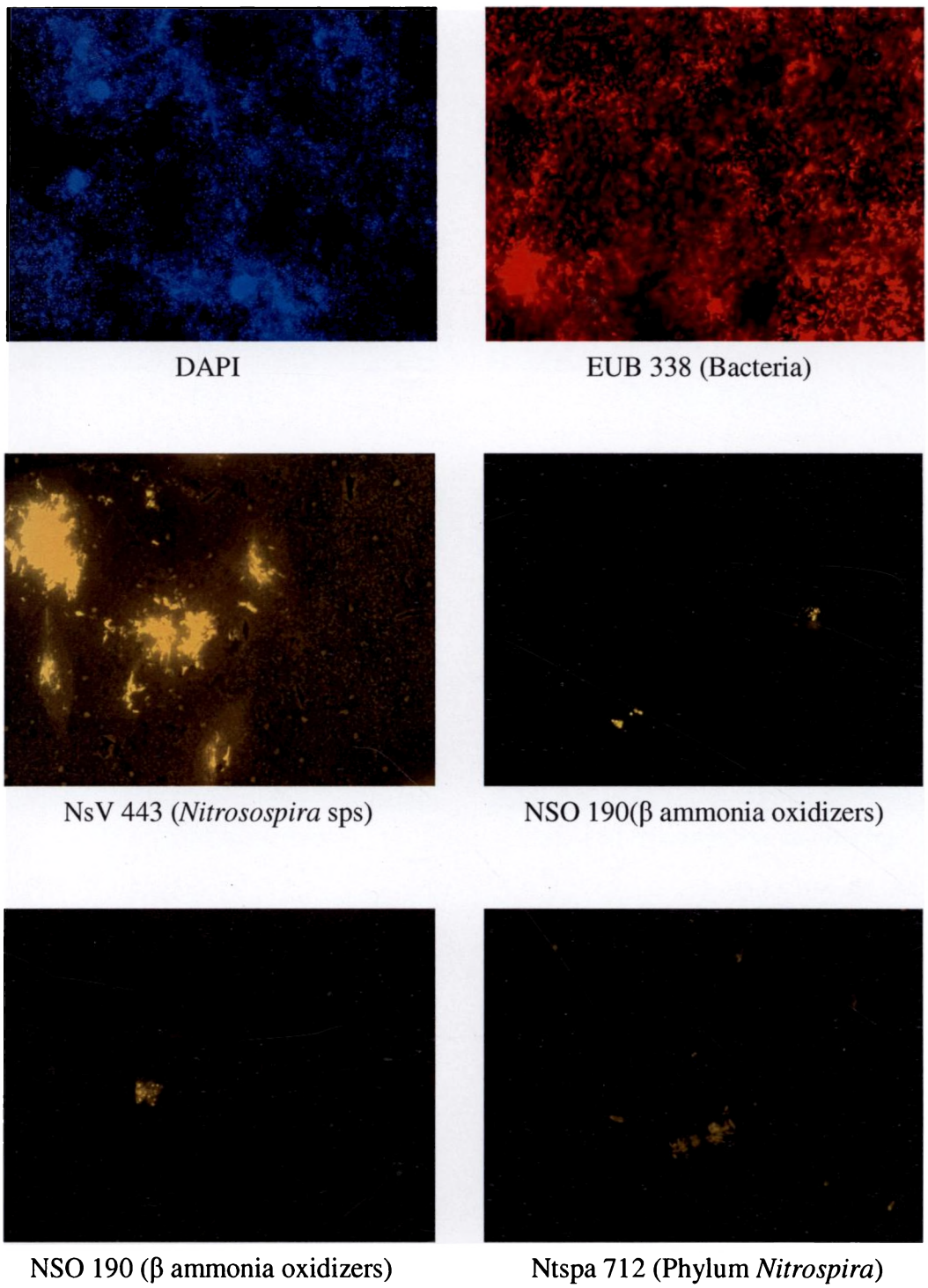


Fig.3. Fluorescence insitu hybridization of the 30 ppt ammonia oxidizing consortia

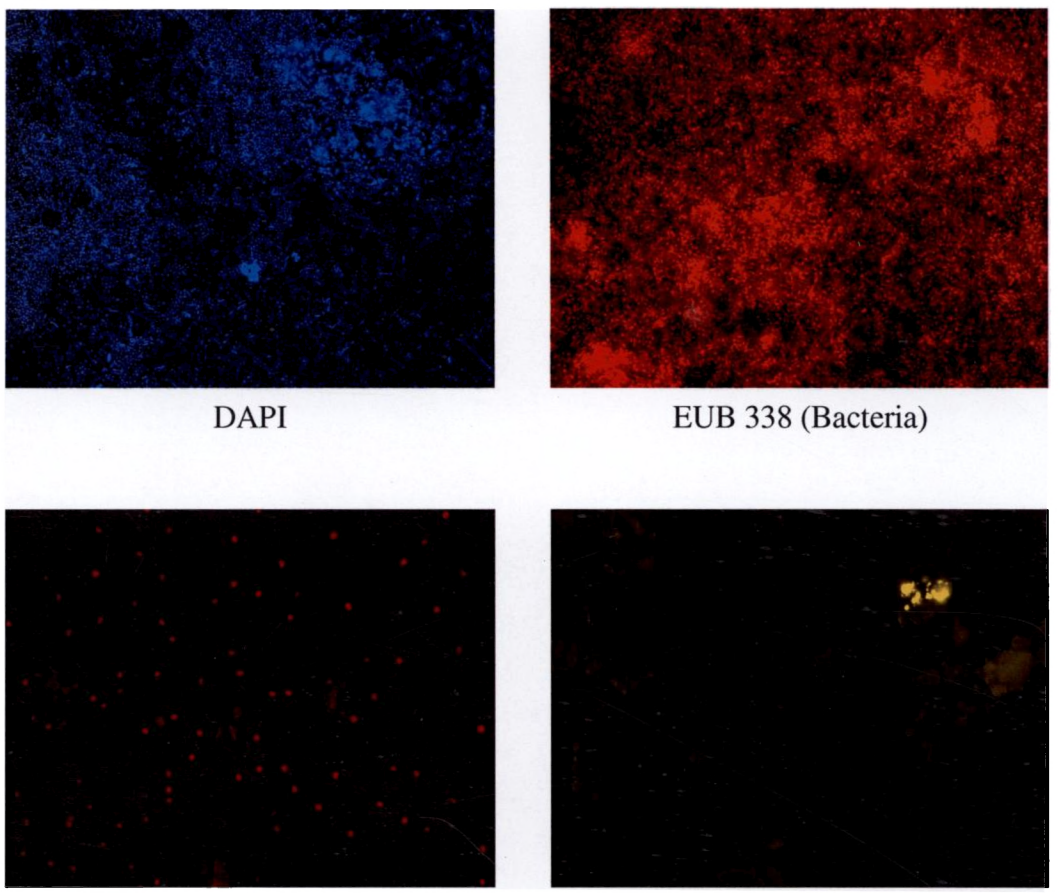


Fig. 4. Fluorescence insitu hybridization of the 15 ppt nitrite oxidizing consortia

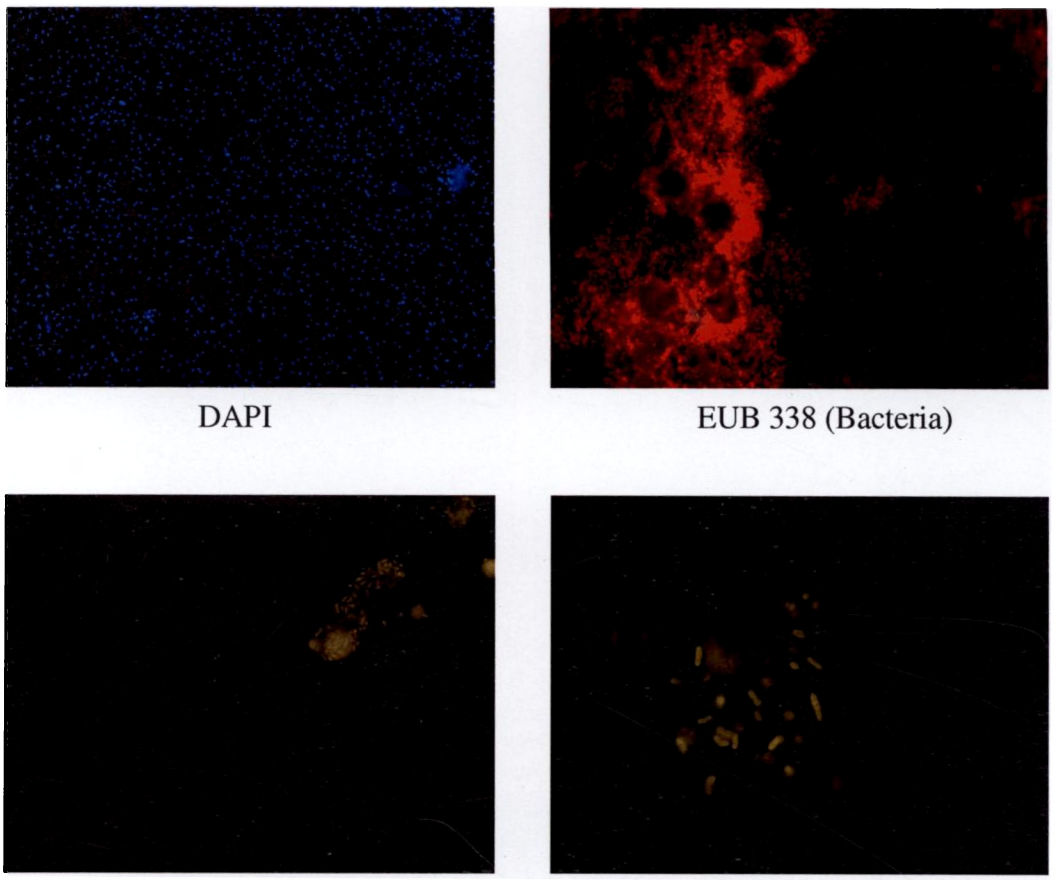


Fig. 5. Fluorescence insitu hybridization of the 30 ppt nitrite oxidizing consortia

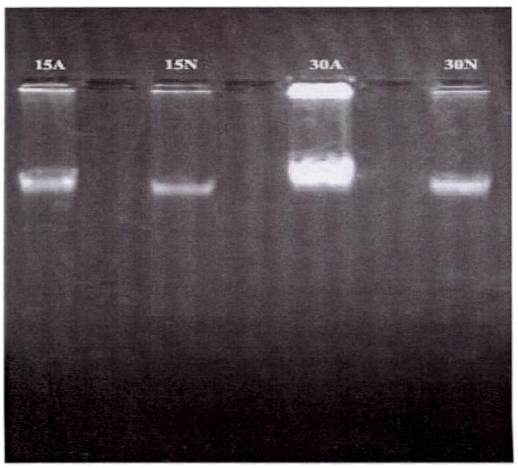


Fig.6. Agarose gel showing the genomic DNA extracted from the nitrifying consortia

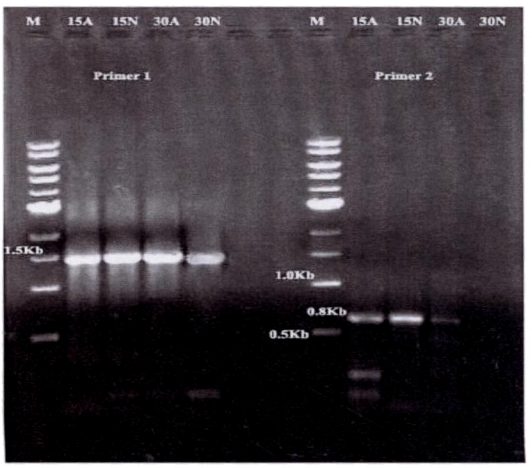


Fig.7. Screening of the primers for amplification of 16S rRNA gene from the nitrifying consortia.

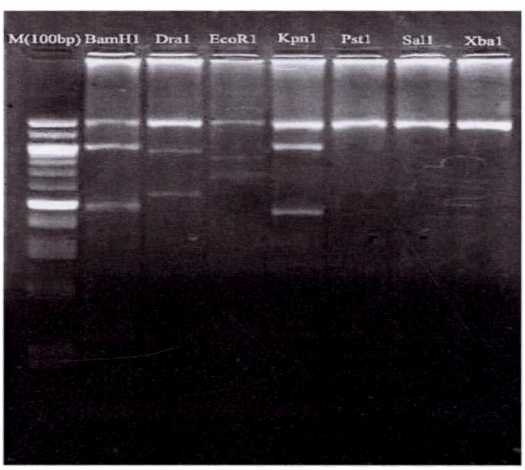


Fig.8. Agarose gel (2%) showing the bands obtained during preliminary screening of the restriction enzymes for ARDRA analysis

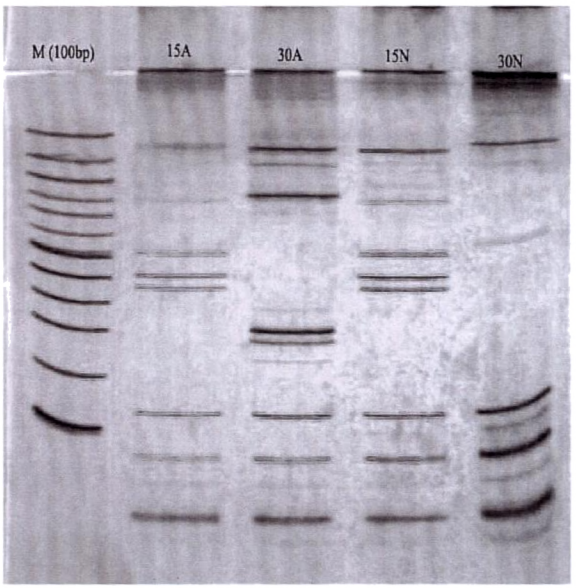


Fig. 9. Amplified ribosomal restriction pattern of the 16S rRNA gene obtained on a 15% SDS PAGE after silver staining with the four nitrifying consortia

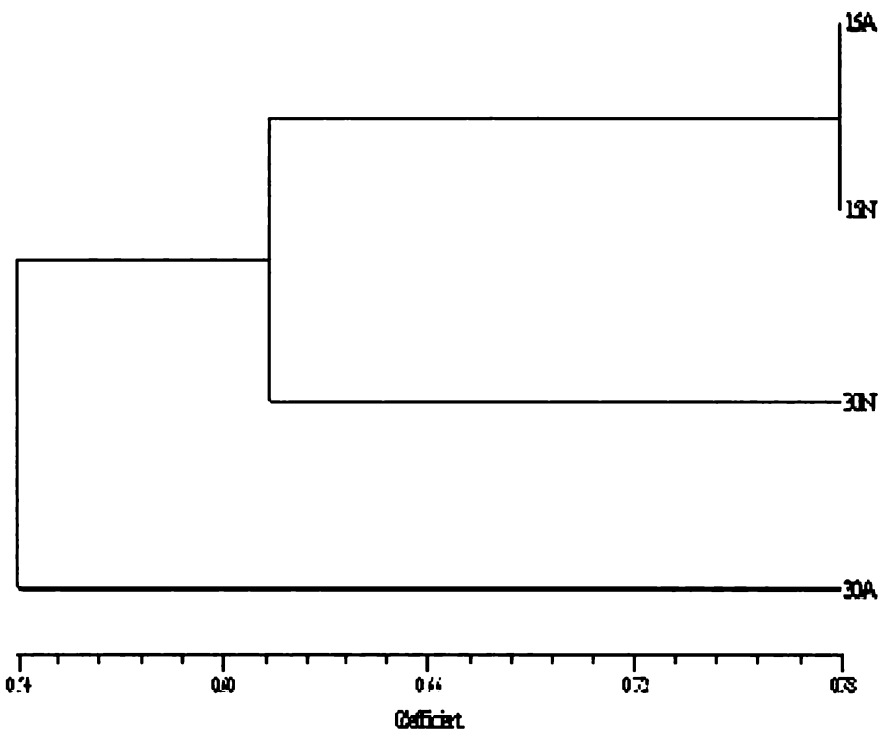


Fig.10. Similarity of the bands obtained with the four nitrifying consortia in the ARDRA analysis

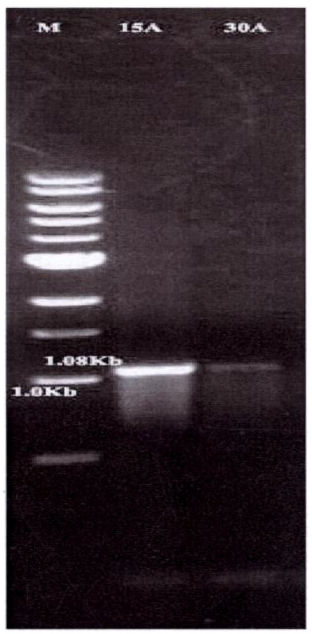


Fig.11. Amplification of β-ammonia oxidizers specific 16S rRNA gene from the nitrifying consortia

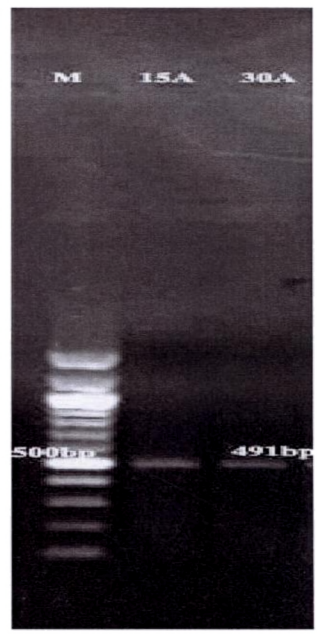


Fig.12. Amplification of the *amo*A gene fragment from the ammonia oxidizing consortia

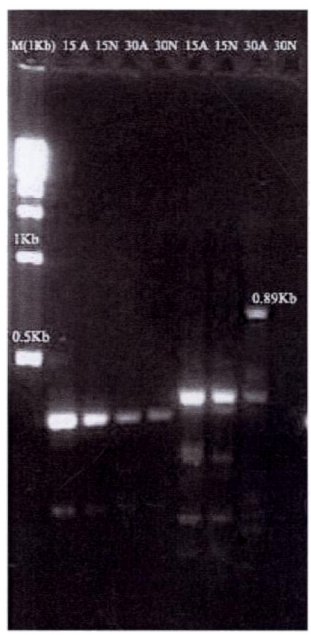


Fig.13. Amplification of *nir*S genes from 30ppt ammonia oxidizing consortia

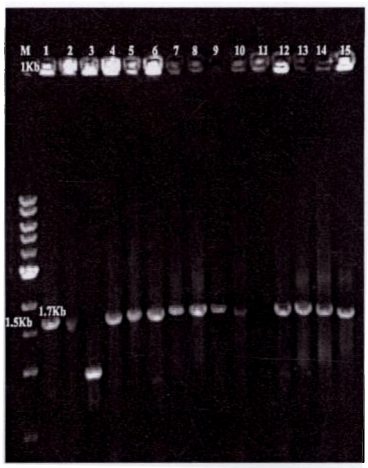


Fig.14. Colony PCR of the clones carrying 16S rRNA gene carrying the desired inserts.

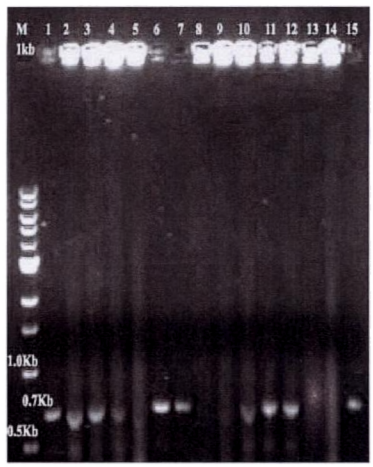


Fig.15. Colony PCR of the clones carrying the *amo*A gene insert

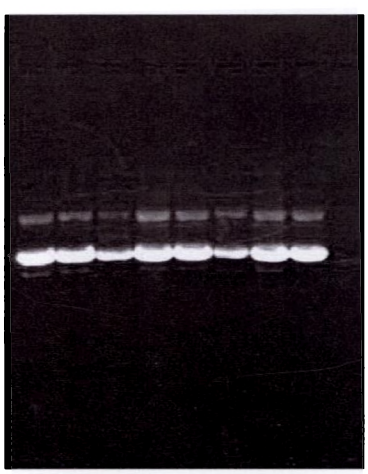


Fig.16. Agarose gel micrograph of plasmids extracted from the positive clones

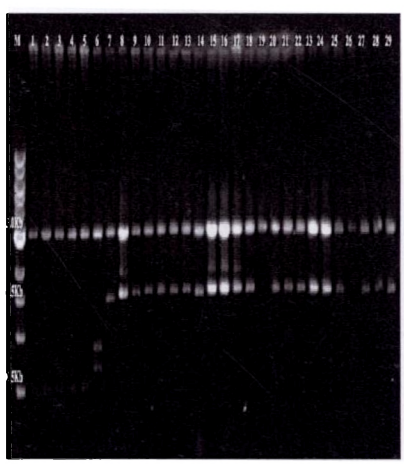


Fig.17. Agarose gel showing the insert released from the plasmids when digested with *Eco*R 1 restriction enzyme

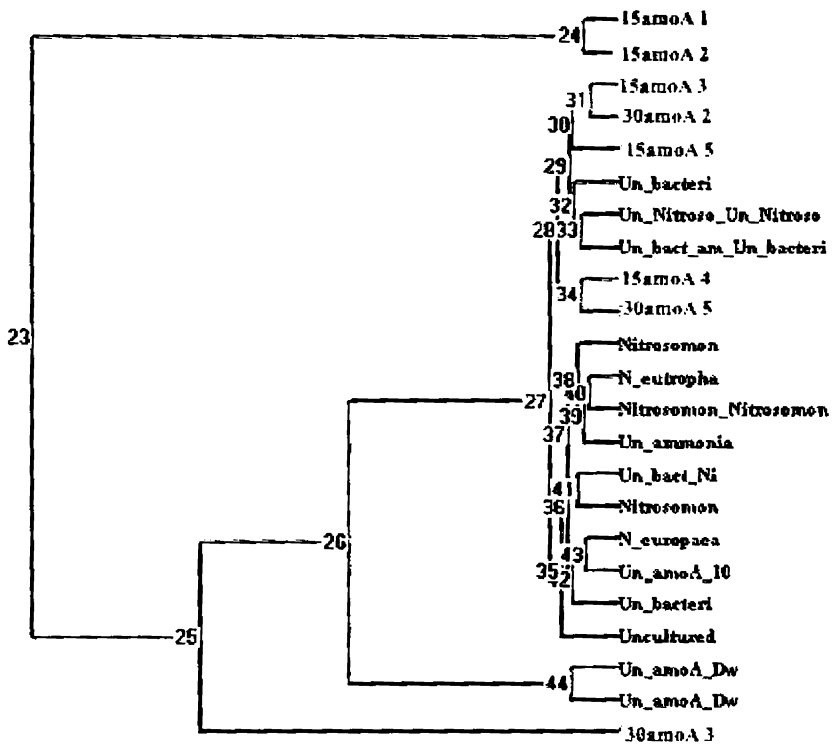


Fig.18. Neighbourhood-Joining consensus phylogenetic tree constructed with the *amo*A gene sequences with their most similar matches in GenBank database. The bootstrap values were fixed at 100. Red high lights indicate the clones from the consortia

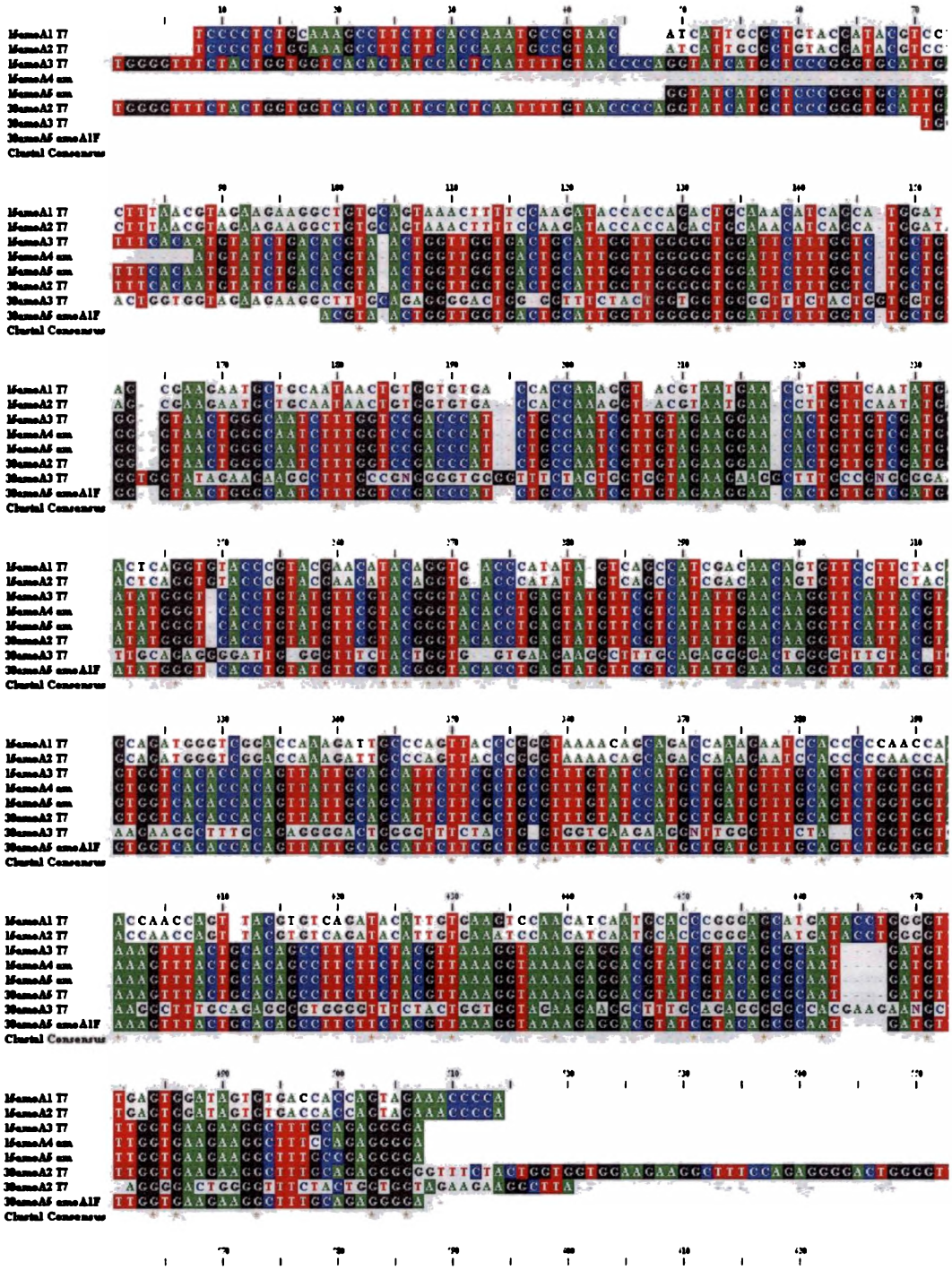


Fig. 19. Multiple aligned sequences of *amo*A gene obtained from 15 and 30 ammonia oxidizing consortia clones. The consensus sequence points are indicated by an asterisk below each alignment.

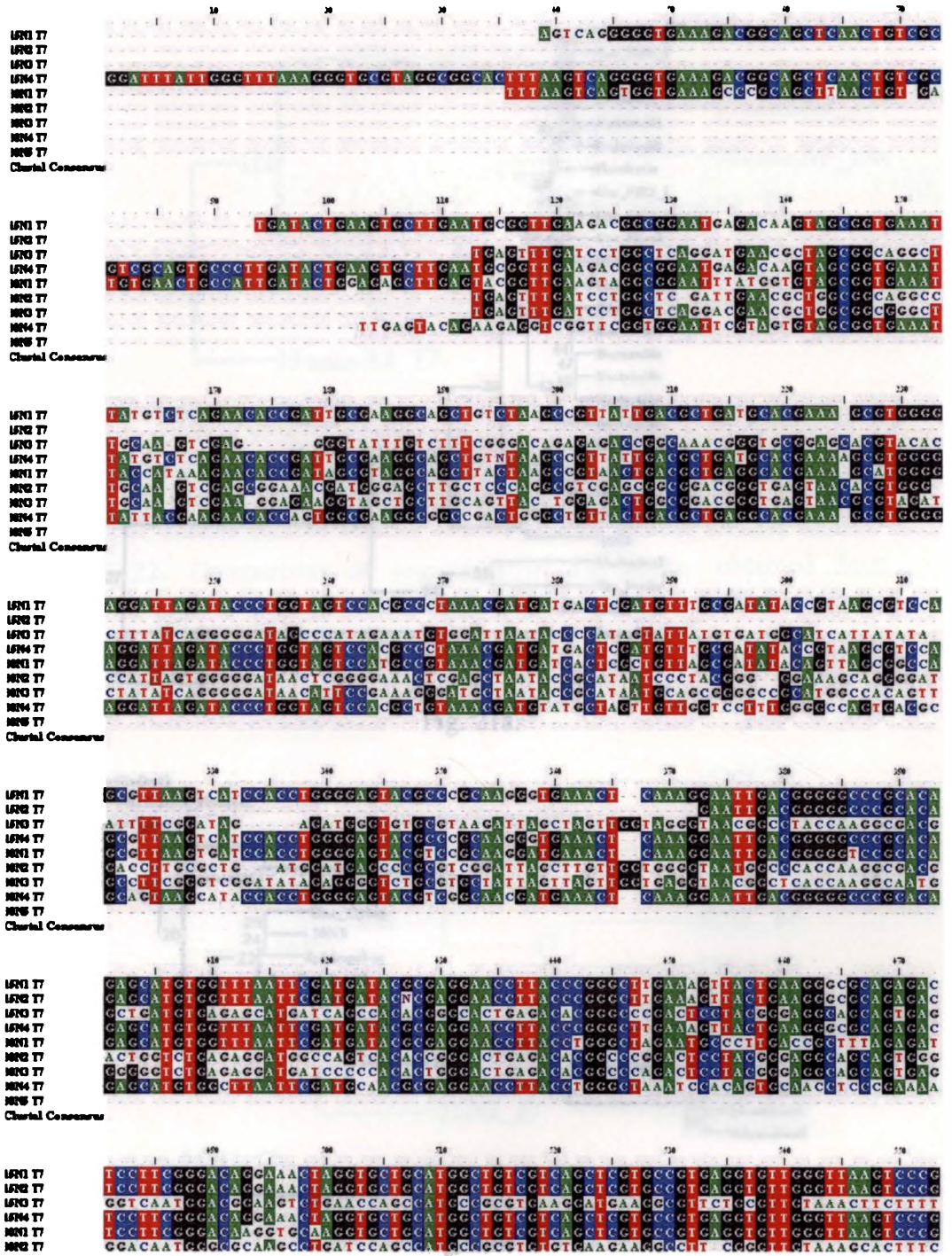


Fig.20. Multiple aligned sequences of 16S rRNA gene obtained from 15 and 30 nitrite oxidizing consortia clones. The consensus sequence points are indicated by an asterisk below each alignment.

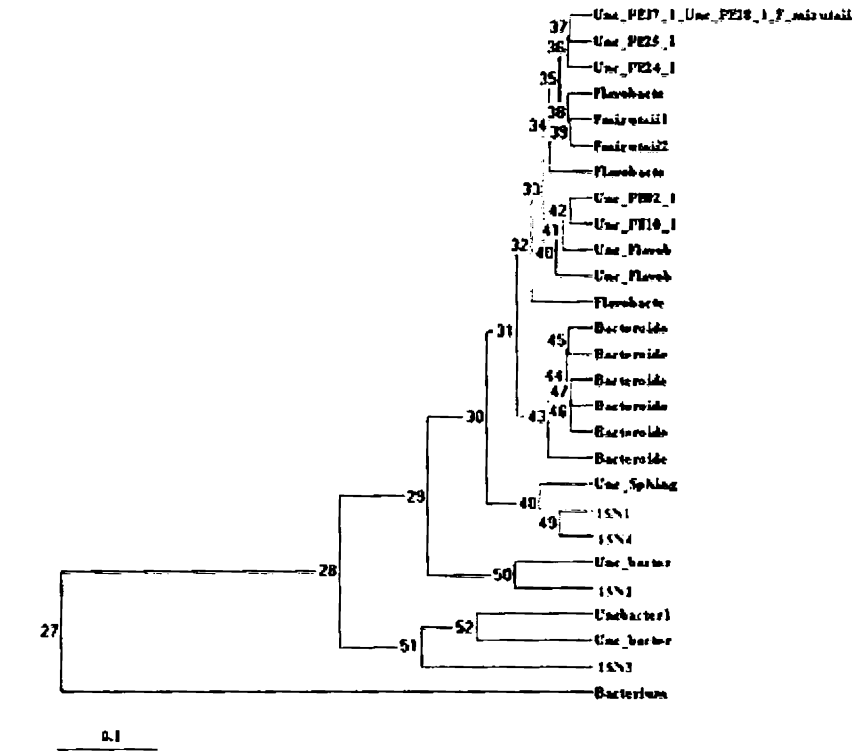


Fig. 21a.

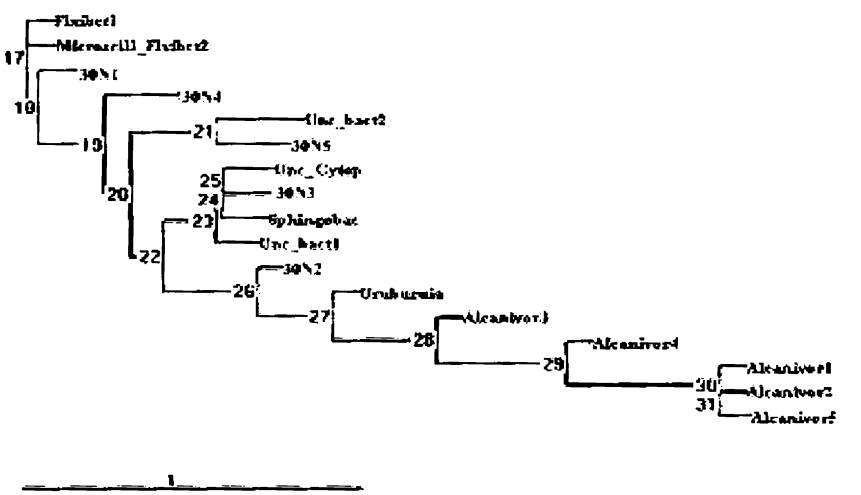


Fig. 21b.

Fig. 21. a and b. Neighbourhood-Joining consensus phylogenetic tree constructed with the 16S rRNA gene sequences from 15N (Fig. 20a) and 30N (Fig. 20b) with their most similar matches in GenBank database. The bootstrap values were fixed at 100. Blue high lights indicate the clones from the consortia.

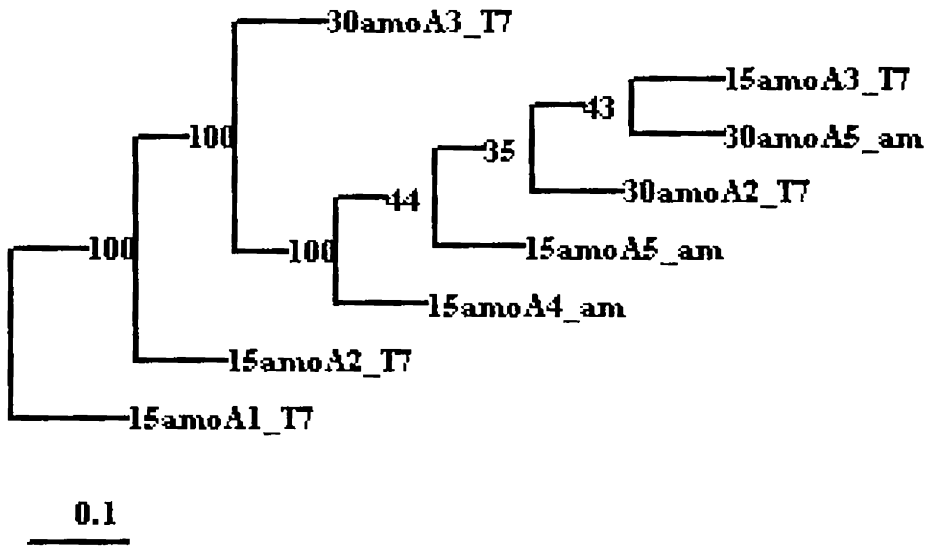


Fig.22. Comparison of sequences of the clones obtained from 15 and 30 ppt ammonia oxidizing consortia with *amo*A gene. The bootstrap values were fixed at 100.

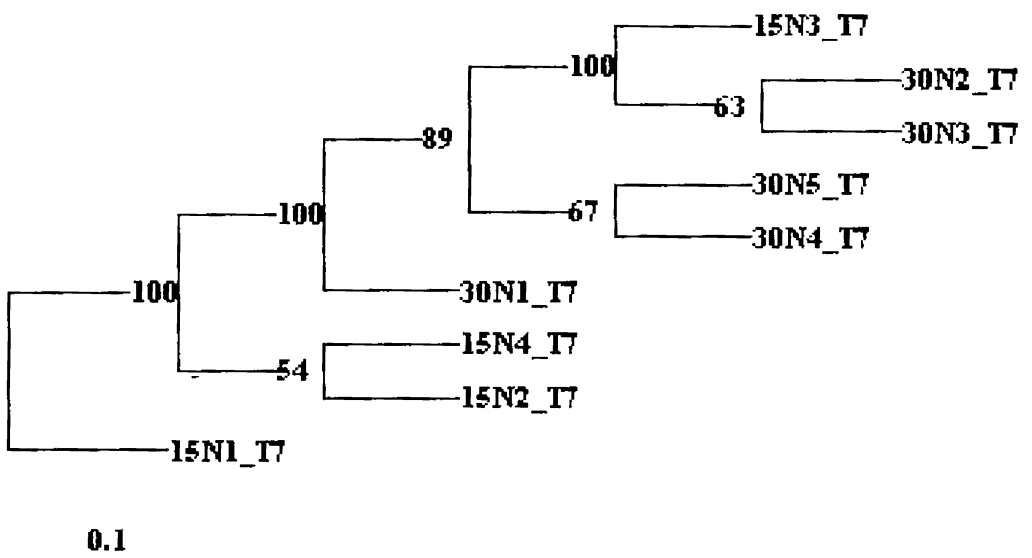


Fig.23. Comparison of sequences of the clones obtained from 15 and 30 ppt nitrite oxidizing consortia with 16S rRNA gene. The bootstrap values were fixed at 100.

Chapter 3

MASS PRODUCTION OF THE NITRIFYING BACTERIAL CONSORTIA

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3.1 Introduction

Cultivation of nitrifying bacteria is hampered by their low growth and poor yield. The shortest generation times measured in laboratory experiments was not less than 7 h for *Nitrosomonas* and 10 h for *Nitrobacter* (Bock et al., 1990). For cell division in natural environments, most nitrifier species even need several days to weeks depending on substrate, oxygen availability, temperature and pH values. This characteristic interferes with cultivation-dependent approaches to investigate number, community composition and dynamics of nitrifiers in different environments. The number of nitrifiers in complex systems has been traditionally determined by the most-probable-number (MPN) technique (Matulewich et al., 1975). However, this method is time-consuming and the nitrifier cell counts determined usually do not correlate well with nitrifying potential estimated for the same environmental sample under optimized laboratory conditions (Belser and Mays, 1982; Mansc and Bock, 1998). These discrepancies illustrate that not all nitrifiers can be cultivated using standard methods (Stephen et al., 1998; Juretschko et al., 1998; Purkhold et al., 2000). Furthermore, in many environments nitrifiers form dense microcolonies of ten to several thousand cells embedded in extracellular polymeric substances. Since these microcolonies are resistant to the dispersal techniques implemented in standard cultivation protocols, the use of these protocols dramatically underestimate the number of nitrifiers occurring in microcolonies (Watson et al., 1989; Stehr et al., 1995; Wagner et al., 1995).

Nitrifying bacteria are autotrophic microorganisms that obtain their energy from the oxidation of reduced nitrogen. The biological process of nitrification involves conversion of toxic ammonia (NH_3) to non-toxic nitrate (NO_3-) through the action of autotrophic nitrifying bacteria.

$$NH_4^+ + 1.5\ O_2 \rightarrow 2\ H^+ + H_2O + NO_2 \quad \text{.....(3.1)}$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3 \quad \text{.....(3.2)}$$

Combining Equations 3-1 and 3-2

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H+ + H_2O \quad \text{.....(3.3)}$$

On adding cell growth to the equation for *Nitrosomonas*, when NH_4^+ is the basis,

$$NH_4^+ + 2.457\ O_2 + 6.716\ HCO_3^- \rightarrow 0.114\ C_5H_7O_2N$$
$$+ 2.509\ NO_2^- + 1.036\ H_2O + 6.513\ H_2CO_3, \quad \text{.....(3.4)}$$

and for *Nitrobacter* when NO_2^- is the basis,

$$NO_2^- + 0.001\ NH_4^+ + 0.014\ H_2CO_3 + 0.003\ HCO_3^-$$
$$+ 0.339\ O_2 \rightarrow 0.006\ C_5H_7O_2N + 0.003\ H_2O + 1.348\ NO_3^- \text{.....(3.5)}$$

Combining these two reactions the overall stoichiometry is:

$$NH_4^+ + 3.300\ O_2 + 6.708\ HCO_3^- \rightarrow 0.129\ C_5H_7O_2N$$
$$+ 3.373\ NO_3^- + 1.041\ H_2O + 6.463\ H_2CO_3 \quad \text{.....(3.6)}$$

According to the equation large amount of alkalinity (HCO_3^-) is destroyed during the oxidation of ammonia to nitrate: equivalent to 8.62 mg HCO_3^-/mg NH_4^+-N removed. The vast majority of the alkalinity utilization is associated with neutralization of the hydrogen ions released during the oxidation of ammonia-N. Only a small part of alkalinity is incorporated into the cell material. The equations also tell us that considerable oxygen is required for nitrification: which is equivalent to 4.33 mg O_2/ mg of NH_4^+-N oxidized to nitrate-N. Of that amount 3.22 mg O_2 will be used by *Nitrosomonas* and 1.11 will be used by *Nitrobacter*. The oxygen requirement of the nitrifying bacteria can have a significant impact on the total amount of oxygen required by a biochemical operation. It can be seen that relatively little biomass will be formed, reflecting the low yields associated with autotrophic growth. For every mg of NH_4^+ removed, only 0.129 mg of biomass will be formed, which is equivalent to 0.166 mg biomass/NH_4^+-N removed. Most of that, 0.146 mg biomass/mg NH_4^+-N removed will be due to the growth of *Nitrosomonas* and only 0.020mg biomass/mg NH_4^+-N removed will be due to *Nitrobacter*. Overall, the growth of nitrifying bacteria will have little impact on the quantity of biomass in a biochemical operation, but will have a large impact on the oxygen and alkalinity requirements (Grady et al., 1999). For

autotrophic biomass growth, yield is often expressed as the mass of biomass COD formed per mass of inorganic element oxidized (Grady et al., 1999) and true growth yields (YA) of 0.212mg biomass COD formed/mg N oxidized by *Nitrosomonas* and 0.0286 mg biomass COD formed/mg N oxidized by *Nitrobacter.* The yield of nitrifiers is less than half that of most heterotrophic bacteria.

Techniques for measuring growth of nitrifying bacteria differ significantly from those used for heterotrophs (Prosser, 1989). Measurement of biomass by absorbance is difficult due to the low growth yields and viable cell counts are inaccurate and require long period of incubation. Consequently, the growth of nitrifiers is usually measured by either substrate utilization or product formation. Mathematical models for design and operation of biological wastewater treatment processes that remove nitrogen do not measure nitrifying bacterial growth rates because they focus on metabolic activity (Henze et al., 2000). The measurement of the changing concentrations of ammonia and nitrate are often used to estimate rates of nitrification (Nogueira et al., 2002; Welsh and Castadelli, 2004). In wastewater treatment, interpreting data from traditional tests (respirations, batch kinetic tests) is straightforward as it is done within a well-defined framework of modeling terms but also have limits (Sin et al., 2005). Different methods give different views of the nitrification process. The activated sludge models (Henze et al., 2000) look at metabolic activity. There are studies based on the fact that during exponential growth in a batch culture, a semilogarithmic plot of nitrite or nitrate concentration versus time will be linear with a slope equal to the maximum specific growth rate (Belser and Schmidt, 1980; Keen and Prosser, 1987). The viable cell count of *Nitrosomonas europea* and nitrite formed showed a good correspondence (Engel and Alexander 1958). When the substrate consumption is linked to growth, the number of catabolic units or activities increases with time. Assuming that the initial substrate concentration is greater than the half saturation concentration an increase in activity concomitant with substrate consumption yields a sigmoid kinetics and the Monod growth kinetic parameters can then be

estimated by nonlinear regression (Robinson and Tiedja JM, 1983). The extent of nitrification in a batch reactor is directly related to the amount of NH_3-N oxidized in the process, however, calculation of μ_A from it is limited, since ammonia nitrogen also serves as the basic nitrogen source and incorporated into biomass at the same time as it is oxidized. Consequently, the concentration of the oxidised nitrogen, NO_X-N, is a much more convenient parameter for this purpose, mainly because NO_X-N is the only model component solely related to autotrophic growth.

The relationship between microbial specific growth rate and the concentration of growth limiting substrate S is generally described by Monod equation (Monod, 1942)

$$\mu = \mu^{max} S / Ks+S \quad \text{.....(3.7)}$$

μ = specific growth rate/day

μ_{max} = max specific growth rate/day

S = substrate concentration mg/L NH_4-N or NO_2-N

Ks = half-saturation constant mg/L NH_4-N or NO_2-N

In general, methods that have been used to estimate kinetic parameters for use in continuous-flow biological reactor modeling can be divided as follows: batch reactor method (Rebac et al., 1999; Huang et al., 2003), chemostat method (Contreras et al., 2000; Park and Noguera, 2004), and continuous stirred tank reactor (CSTR) method (Dincer and Kargi, 2000; Latkar et al., 2003; Bhat et al., 2006). Among these methods, the batch reactor method is often used to estimate kinetic parameters because this method only needs a short operation time (i.e., several hours to a few days). Nonetheless, when the batch reactor method is used, a rather high initial limiting-substrate concentration in the batch reactor is required to give sufficient degradation data points for regressive analysis for kinetic parameters. Such a sudden change of high substrate concentration in the batch reactor (i.e., compared with a relatively low residual substrate concentration in a continuous-flow biological reactor system) inevitably stimulates microbial cells to synthesize RNA and enzymes that are required for the new growth environments,

resulting in sluggish physiological adaptation and biochemical reaction of bacterial cells (Iyer et al., 1999).

The maximum specific growth rate coefficient for the autotrophic bacteria are considerably less than those for heterotrophic bacteria, reflecting their more restricted energy yielding metabolism and the fact that they must synthesize all cell components from carbon dioxide. This suggests that special consideration must be given to their requirements during design of reactors in which both carbon oxidation and nitrification are to occur. Although half- saturation coefficients for the autotrophs are less than the reported values for heterotrophs growing on complex substrate, they are similar to the values reported for heterotrophs growing on single organic compounds. As a consequence of their small size, the kinetics of nitrification will behave in a zero order manner over a broad range of ammonia and nitrite concentrations; this has a significant impact on bioreactor performance (Grady et al., 1999) A major difference in the growth characteristics of heterotrophic and autotrophic biomass is the greater sensitivity of the latter to the concentration of dissolved oxygen. Whereas the value of the half-saturation coefficient for oxygen is very low for heterotrophs, the values for the two genera of autotrophs are sufficiently high in comparison to typical dissolved oxygen concentration.

Values of half saturation coefficients for both *Nitrosomonas* and *Nitrobacter* have been reported to lie between 0.3 and 1.3 mg/L (Sharma and Ahlert, 1977). Measurements which considered the effects of diffusional resistance on half-saturation coefficients have suggested that the true values lie near the lower end of the range (Tanaka et al., 1981; Siegrist and Gujer, 1987), and values of 0.50 and 0.68 have been adopted as typical for *Nitrosomonas* and *Nitribacter*, respectively, in systems in which some diffusional resistance will occur (Rittmann and Snoeyink, 1984).

The primary requirement of a viable mass production process is the optimization of a cost-effective medium. The production of nitrous acid from ammonia constantly decreases the pH and therefore the free ammonia concentration in the

culture broth. Regular pH correction steps are required to neutralize the produced nitrous acid, increasing the pH and the free ammonia concentration in the culture. Typically sodium carbonate, sodium hydroxide or potassium carbonate has been used for pH adjustment (Hynes and Knowles, 1984; Keen and Prosser, 1987; Prosser, 1989). In a study on the cultivation of *N. europaea*, the growth rate and yield was reported to be low, and growth is regulated at various levels in response to specific chemical requirements (Laanbroek et al., 2002; Sayavedra-Soto et al., 1996; Stein and Arp, 1998; Stein et al., 1997), through quorum sensing (Batchelor et al., 1997) and the potential of self-inhibition through the build up of metabolic end products (Stein and Arp, 1998).

There are not many reports on the mass production of autotrophic nitrifying bacteria. Chapman et al. (2006) developed improved methods for the cultivation of the chemolithoautotrophic *Nitrosomonas europaea* in fed batch and continuous cultures at laboratory level. Continuous culture in bioreactors with cell recycle is a relatively new method to produce concentrated cultures of actively growing microbial species that have low growth rates and yields. This technique has been used previously to maintain resting nitrifying bacteria in pure and mixed culture with complete biomass retention (Tappe et al., 1996, 1999; Zart and Bock, 1998). However, to achieve actively growing cell biomass for biotechnological applications, a portion of the biomass must be continuously removed at steady state to maintain growth pressure on the culture.

The attempts to cultivate nitrifying bacteria are more in freshwater applications whereas aquaculture applications require optimum performance at a range of salinities. There are reports that at field level the maximum nitrification capacity of salt water systems was considerably lower than in freshwater systems (Nijhof and Bovendeur, 1990). However, Saucier (1999) was able to obtain nitrification rate similar to freshwater systems under saline conditions. Salt water systems needed much longer start up period (Chen et al., 2006) and adaptation of freshwater biofilter to higher salinities was found to be an option (Nijhof and

Bovendeur, 1990). Instead of acclimating freshwater systems to higher salinities, it is imperative to develop salt water based systems. A few bacterial cultures containing nitrifying bacteria to control the ammonia level in culture water are available commercially and are aimed especially at aquarium hobbyists (Verschuere et al., 2000). Perfettini and Bianchi (1990) used inocula consisting of frozen cells to accelerate the conditioning of new closed seawater culture systems. A nitrifying consortia cultivated in batch culture (Koops et al., 2004) was found to be effective in nitrification on applying to pond water. Meanwhile for setting up instant nitrification in tropical shrimp and prawn hatcheries two ammonia and two nitrite oxidizing consortia have been developed by enrichment method form shrimp culture systems (Achuthan et al., 2006). These consortia were further immobilized in nitrifying bioreactors developed (Patent Application No. 828/DEL/2000 of 13.9.2000) to achieve rapid biological nitrification in shrimp/prawn hatcheries (Singh et al., 2007). The reactors, Stringed Bed Suspended Bioreactors (SBSBRs) and Packed Bed Bioreactors (PBBRs), function on the nitrifying biofilms generated by immobilizing the above consortia on plastic beads assembled in the form of cartridges. For activation of the commercialized reactors large quantities of nitrifying bacterial consortia are required. As first phase in the mass production the consortia were amplified in a 2L fermentor under optimized conditions to generate start up culture for mass cultivation. As the next phase, a large volume fermentor was designed, fabricated with locally available materials and demonstrated for mass production of the selected nitrifying bacterial consortia.

3.2 Materials and methods

3.2.1 Optimum growth requirements of the nitrifying bacterial consortia

Fermentation process for mass production of the nitrifying bacterial consortia was designed based on their optimum growth requirements (Table-1).

3.2.2 Activation of stored nitrifying bacterial consortia

The nitrifying bacterial consortia maintained in refrigerator were brought to room temperature and required aliquots were kept over a rotary shaker at 100 rpm under

obscurity. Equal volumes of growth media (sea water-100mL, $(NH_4)_2SO_4$/ $NaNO_2$ - 10µg, KH_2PO_4 - 2µg) as per Watson (1965) were added to the cultures in order to reduce the product inhibition. Daily monitoring was done to check the quantity of the substrate consumed (NH_4-N/ NO_2-N) and product formed (NO_2-N/ NO_3-N N). Drop in pH, when observed in the case of ammonia oxidizers, was compensated using sodium carbonate. Sterile distilled water was added to make up evaporation loss.

3.2.3 Amplification of the nitrifying bacterial consortia in 2L fermentor

A 2L capacity baby fermentor (New Brunswick, USA, Bioflow 2000) (Fig.1) was used for amplifying the four ammonia oxidizing and nitrite oxidizing consortia. The media according to Watson (1965) prepared in 15ppt and 30ppt seawater was used for mass production of the consortia meant for non-penaeid (AMONPCU-1 and NIONPCU-1) and penaeid (AMOPCU-1 and NIONPCU-1) hatcheries respectively. The detachable fermentor vessel with the medium was autoclaved at 10 lbs for 10 minutes and reassembled with control panel. Optimum pH and temperature (Table-1) were put under auto-adjustment mode and the rotation was set at 200rpm with an airflow rate of 1.0 L min^{-1}. Inocula were drawn from the cultures that were activated over rotary shaker. The fermentor vessel was covered with black cloth to prevent entry of light. The substrate consumed (NH_4-N/ NO_2-N) and product formed (NO_2-N/ NO_3-N N) were estimated once in 24h. As and when pH dropped, it was automatically adjusted by addition of 10% Na_2CO_3 through the base port. With consumption of the substrates, NH_4^+-N and NO_2^-N, they were supplemented with fresh aliquots at an exponential rate but with 10µg mL^{-1} at a time. On attaining stationary phase characterized by decline in the production and consumption rate of NO_2^-N in the ammonia and nitrite oxidizing consortia respectively, they were harvested fully and used for inoculating the 200L fermentor. The initial and final biomass was estimated gravimeterically. The μ^{max} for each of the consortia at 28°C was estimated using a mathematical model of the quadratic form fitted into the experimental data on product formed (NO_2-N for ammonia oxidizers and NO_3-N for nitrite oxidizers) by the method of least

squares using the statistical software SPSS (SPSS Inc., USA). The equations obtained below were solved for estimating the μ^{max} values.

AMONPCU-1

$y = -0.958247+0.253188x-0.00462x^2$ (R^2 0.91; $P<0.05$)..... (3.8)

NIONPCU-1

$y = -0.93007+0.257609x-0.004276x^2$ (R^2 0.97; $P<0.0001$).....(3.9)

AMOPCU-1

$y = -0.430360+0.199245x-0.003181x^2$ (R^2 0.99; $P<0.0001$) .....(3.10)

NIOPCU-1

$y = -0.537170+0.172404x-0.002370x^2$ (R^2 0.94; $P<0.0001$).....(3.11)

3.2.4 Designing and fabrication of nitrifying bacterial consortia production unit

The nitrifying bacterial consortia production unit (NBCPU) was designed and fabricated with locally available components taking in to consideration of the requirements for getting maximum biomass within the shortest duration possible. The NBCPU has fermentation vessels consisting of 200L wide mouthed carboy made of polyethylene (PE) fixed with a central 0.5 H.P AC/DC agitator motor which works on 500 W current consuming 1.5 units electricity per day. Speed of the motor is adjustable from 0-500 rpm. Inside the vessel a stainless steel pipe goes wound round as coil, through which water gets circulated at constant temperature from a thermocirculator, which works at 2500 W, consuming 3 units per day to maintain the optimum temperature. Separate provisions are given for insertion of a temperature probe and for addition of medium and inoculum. Aeration is provided through a stainless steel pipe, which delivers air at the centre of the bottom through an air sparger. A drain pipe is placed at the base of the tank. For preparing media, a boiler (5000W, stainless steel) is arranged associated with the facility where from the boiled media can be passed through a sterile pipe to the fermentor (Fig.2 and 3)

3.2.5 Mass production of nitrifying consortia (AMOPCU-1 and AMONPCU-1) for penaeid and non-penaeid hatchery system in 200L fermentor

The 200 Litre fermentor vessel of NBPCU was rinsed thoroughly with 70% ethanol (1 L) and after half an hour washed with sterile (autoclaved) tap water and drained off. Mass production of the consortia AMOPCU-1 and AMONPCU-1 for penaeid and non-penaeid hatchery system respectively was optimized in NBCPU. For the culture seawater of respective salinity was sterilized in the 100L capacity boiler, and transferred to the fermentor vessel through sterile tubing. It was aerated for 2 days and plated out on to ZoBell's agar and Saboraud dextrose agar prepared in aged seawater to record the total viable bacterial and fungal populations present if at all any, before inoculating with the nitrifiers. Optimum pH, temperature and substrate concentrations were maintained as given in Table-1 during the culture period. pH was adjusted manually using aqueous 10% sodium bicarbonate and temperature regulated to 28°C by appropriate adjustments in the thermocirculator. Inocula were added to a final concentration of 1% (v/v) and incubated under agitation at a rate of 100rpm and with continuous aeration at 1Lminute^{-1}. The initial biomass was measured gravimetrically. The substrates consumed (NH_4-N/ NO_2-N) and product formed (NO_2-N/ NO_3-N N) were monitored daily as above. Whenever pH was altered it was adjusted to optimum manually using 10% Na_2CO_3. As the consumption of NH_4^+-N progressed; it was supplemented with fresh aliquots of substrates at an exponential rate. This process was continued until the cultures attained stationary phase. Based on the data generated, growth pattern, substrate consumption and product formation, alkalinity destruction and yield coefficient were determined.

3.2.6 Alkalinity destruction

pH was monitored daily using a pH probe (Scientific Tech- India) and adjusted to optimum level using 10% Na_2CO_3. Alkalinity (APHA, 1998) destruction due to hydroxylamine production by ammonia oxidizers were calculated based on the relationship, 6.0-7.4 mg alkalinity was destroyed per milligram NH_4^+- N oxidized to NO_2^-- N (EPA, 1975).

3.2.7 Analyses

The substrate/product levels were determined daily by estimating ammonia (TAN) (Solarzano 1969), nitrite (NO_2-N) (Bendschneider and Robinson 1952) and nitrate (NO_3-N) (Strickland and Parsons, 1972).

3.2.8 Yield coefficient/cell yield 'Y' of the consortia

Yield coefficient, 'Y' was determined as the ratio of weight of cells / biomass generated to the weight of substrate oxidized or the ratio of the quantity of product formed to the quantity of substrate utilized following Sharma and Ahlert (1977). For determining the cell yield or biomass generated a known quantity of the culture was passed through pre-weighed membrane of 0.22μm pore size, dried at 80°C overnight and stabilized in desiccators and weighed to find the difference as the biomass (dry weight).

3.3 Results and Discussion

3.3.1 Activation, amplification and growth of the consortia

As presented in Fig 4, four to six days were required for the cultures to become active after long refrigeration. Uniformly in all consortia, quantity of the product (NO_2^--N and NO_3-N) formed was much higher than that of the substrate (NH_4^+-N and NO_2^--N) consumed. On amplification in a 2L fermentor the substrate consumed, product formed, and the total biomass generated were worked out sequentially for all the consortia and the product formation was higher in magnitude, than the substrate consumption as could be seen from the growth curves constructed (Fig 5a, 5b, 5c, 5d). Accordingly after 20 to 25 days of culturing, the nitrifiers were found to enter in to near stationary phase characterized by declining in NH_4^+-N consumption and NO_2^--N production and NO_2^--N consumption and NO_3^--N production, with respect to ammonia and nitrite oxidizers. The non-linear regression analyses of the log NO_2-N/NO_3-N data yielded maximum specific growth rates for the consortia (Table 3) as AMONPCU-1: 0.105/hr; NIONPCU-1: 0. 123/hr; AMOPCU-1: 0.112/hr and NIOPCU-1: 0.11/hr during growth in 2L fermentor (Table 3). The net biomass

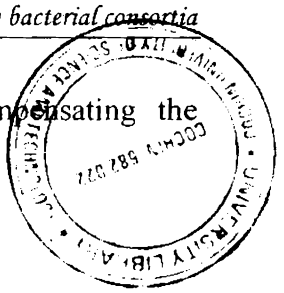
yield of the consortia after 25 days in 2 L fermentor were AMONPCU-1- 157 g/L, AMOPCU-1- 159 g/L, NIONPCU-1- 140 g/L and NIOPCU- 156 g/L respectively. The yield coefficient (g NO_2-N g NH_4-N^{-1} or g NO_3-N g NO_2N^{-1}) of AMONPCU-1, AMOPCU-1, NIONPCU-1 and NIOPCU-1 were 1.315, 2.08, 1.6 and 2.4 respectively (Table 3).

3.3.2 Mass production of the consortia in 200L fermentor

The substrate consumption and product formation during the growth of AMOPCU-1 and AMONPCU-1 in 200L NBCPU are given in Fig 6a and 6b. The systems which started with 10 mg mL^{-1} residual NH_4^+-N could consume 1242 and 1948 mg NH_4^+-N L^{-1} over a period of 270 and 160 days respectively with a total corresponding output of 1525 and 1300 mg NO_3^-N L^{-1}. From the growth curve of AMOPCU-1 it was inferred that until 70 days of incubation there was progressive build up of NO_2^--N. Subsequently within 20 days it rapidly declined and nitrate started getting accumulated. After 90 days no residual nitrite could be detected and NH_4^+-N was found to be oxidized straight away to NO_3^--N. In a similar pattern, AMONPCU-1 exhibited progressive build up of NO_2^--N during the first 30 days and subsequently with in another 20 days it rapidly declined concomitant with the building up of nitrate. No residual nitrite could be detected suggesting a situation similar to that of AMOPCU-1.

3.3.3 Alkalinity destruction

The quantity of Na_2CO_3 (10% aqueous) required to compensate the lowering pH and alkalinity, and to bring them back to optimum during mass production of the consortia in 200L fermentor was estimated (Table.2). While the total NH_4^+-N removed by AMOPCU-1 and AMONPCU-1 was 1.242 and 1.948 g/L during the culture period of 270 and 160 days respectively, the total destruction of alkalinity was by 7.452-9.190 and 11.688 – 14.415 g/L respectively. To compensate the alkalinity destruction, the consortia AMOPCU-1 and AMONPCU-1 were fed with 62 and 78 g Na_2CO_3 respectively during the period of culture. Clearly



AMONPCU-1 demanded higher quantity of Na_2CO_3 for compensating the alkalinity destroyed.

3.3.4 Yield coefficient and biomass

Since the consortia on mass production had attained equilibrium in the two step process of nitrification by converting $NH4^+$-N directly to NO_3^-N, with out permitting residual NO_2^--N to detectable levels, the yield coefficient in this case was calculated from the quantity of NO_3^--N produced (Table 3). On attaining stationary phase yield coefficient of AMOPCU-1 was found to be 1.23 and that of AMONPCU-1 0.67. The net biomass yield of AMONPCU-1 at the end 160 days culture in 200L fermentor was 125.3 g/L and that of AMOPCU-1 at the end of 270 days were 165 g/L.

3.4 Discussion

The mass production nitrifying bacterial cultures has been challenging owing to their slow growth rates (Nejidat and Abelovich, 1994; Voytek and Ward, 1995) making turbidometric growth detection difficult. Nitrifiers are typically cultured in weakly buffered media with phenol red as indicator to monitor growth by acidification. The decreasing pH reduces the viable energy source and excess acid is neutralized with sodium carbonate or sodium hydroxide (Prosser, 1989). Though there are few reports on the cultivation of pure cultures of nitrifiers (Tappe et al., 1996; Zart and Bock 1998; Chapman et al., 2006), attempts were never made to scale up the process to large volumes. In this regard, this is the first report of a mass production of nitrifying consortia under saline conditions for industrial applications.

An important consideration for the designing of the mass production process was cost-effectiveness. The medium optimized here was sea water based and required only the addition of substrates and carbonate to maintain optimum pH. For the design of 200L fermentor locally available materials were used. The fermentation tanks have been made opaque and placed well protected from sunlight, because the visible and UV light rays are lethal to nitrifying organisms (Muller- Negluck and Engel, 1961, Johnstone and Jone, 1988; Diab and Shilo, 1988b).

Initial process in the mass production of nitrifying consortia was the activation of cultures stored at 4^0C by amplification in a 2L fermentor to generate sufficient inocula for cultivation in 200L fermentor. While 4 to 6 days were required for activation, amplification demanded 20 to 25 days. In both these processes the quantity of the product formed was considerably higher than the substrate consumed as observed in our previous studies with nitrifying bacterial consortia generated from other sources (Ramachandran, 1998). The yield coefficients of the all the four consortia were higher during the activation. The maximum specific growth rate of the ammonia oxidizers at AMONPCU-1 (30ppt) and AMOPCU-1 (15 ppt) were 0.112 hr^{-1} and 0.105 hr^{-1}) whereas those for NIONPCU-1 (30ppt) and NIOPCU-1 (15ppt) were 0.11 $hr^{-1}$and 0.123 hr^{-1} respectively. The maximum specific growth rate coefficient for *Nitrosomonas* has been reported to lie between 0.014 (Lawrence and McCarty, 1970) and 0.092 (Sharma and Ahlert, 1977) hr^{-1}, with a value of 0.032 hr^{-1} considered to be typical at 20 ° C (Rittmann and Snoeyink, 1984). The maximum specific growth rate coefficient for *Nitrobacter* is similar to that for Nitrosomonas, having reported to lie between 0.006 (Lawrence and McCarty, 1970)) and 0.060 (Sharma and Ahlert, 1977) hr^{-1}. Likewise, the value considered being typical, 0.034 hr^{-1} is similar to that for *Nitrosomonas*. The maximum specific growth rate of all the four consortia was found to be higher than the reported values. Temperature has a strong affect on the growth of nitrifiers (USEPA, 1993). The maximum growth rate at 30°C is two or three times higher than at 20°C. In the present study the consortia were cultured at 28°C and that may the reason for higher values compared to reported values where experiments were carried out at lower temperatures. However, the values reported ammonia oxidizers at higher temperatures were also found to be lower than the present values; 0.043 hr^{-1} at 30°C (Vadivelu et al., 2006), 0.042 hr^{-1} at 35°C (Van Hulle et al., 2004) and 0.039 hr^{-1} at 30°C (Keen and Prosser (1987). The large variation in the reported values is likely due to the use different strains and growth conditions in these studies (Vadivelu et al., 2006)

The nitrifying bacterial consortia production unit (NBCPU) designed and fabricated was successfully run in mass production of ammonia oxidizing consortia at 30 and

15 ppt salinities (AMOPCU-1 and AMONPCU-1) with nitrite oxidation and building up of nitrate in the fermentor with both the consortia establishing a two step nitrification process. It is an accepted principle that when populations of nitrifying bacteria get established under steady state conditions residual nitrite shall be too low to be detected with progressive building up of nitrate (Gray, 1990). Similar observations were also made by Achuthan et al. (2006) during the enrichment of the consortia from shrimp ponds. It has also been established that oxidation of nitrite to nitrate is more rapid than the preceding step (Stesel and Barnard, 1992). This was especially true when the consortia AMOPCU-1 and AMONPCU-1 were grown in NBCPU as no residual nitrite could be detected after 90 days of culturing establishing the existence of a population of nitritifiers and nitratifiers in equilibrium. It has been observed in our study that there exist sharp correlation between the decrease of nitrite in the bulk fluid and disappearance of suspended cells to form biofilm. This is in agreement with Bovendeur (1989) who demonstrated that nitratification is slightly delayed and is subsequent to the development of nitritification capability. Ammonia oxidizers of the genus *Nitrosomonas* seem to capable to nitrification and simultaneous dentirification with ammonia serving as electron donor (Zart and Bock, 1998). If this metabolism could be handled technically there would be no need for a large scale separation of nitrification or denitrification or supply external substrate for denitrifcation. The successful mass production process of a single consortium of either AMOPCU-1 or AMONPCU-1 having the nitritifiers and nitratifiers in equilibrium is highly advantageous in practical sense as activation of the nitrifying bioreactors can be achieved for total conversion of ammonia to nitrate with a single set of reactors.

During this production process the cultures AMOPCU-1 and AMONPCU-1 consumed comparatively lesser quantity of Na_2CO_3 (62 and 78 g respectively) for compensating the alkalinity destruction. Obviously this has not been adding much to the cost of production. There are reports that carbonate based pH control yielded less cell density of *Nitrosomonas europea* (Chapman et al., 2006) compared to

bicarbonate fed batch cultures. However, in the present study the addition of carbonate was sufficient to yield high biomass.

Yield coefficient of AMOPCU-1 (1.23) and AMONPCU-1 (0.67) on mass production were comparatively higher to the estimated value (0.2) based on the thermodynamics of growth of ammonia oxidizers. In the practical sense also (Sharma and Ahlert, 1977) there has been reports of very low yield coefficient for ammonia (0.03-0.13) and nitrite (0.02-0.08) oxidizers. This is indicative of the better energy conversion efficiency and higher carbon dioxide fixation potentials of AMOPCU-1 and AMONPCU-1, primarily as the characteristics of tropical organisms and secondarily due to their mixed culture configuration. Ramachandran (1998) also while experimenting with nitrifying consortia developed from sewage in India had experienced a similar comparatively higher yield coefficient (0.1864 to 0.1939) for ammonia oxidizers and (0.1745 to 0.1986) nitrite oxidizers.

In the cultivation of *Nitrosomonas europea* pure cultures, fed batch culturing with bicarbonate buffering yielded culture density of 30 mg dry wt/ L whereas continuous culture under energy limited growth conditions in a bench scale bioreactor using a microfiltration membrane for high cell recycle produced final culture densities greater than 350 mg dry wt/ L. However, this cannot be compared to the higher values obtained in the present study where the consortia were cultured for long duration in larger volumes under saline conditions. It should be noted all that all accumulated extracellular organics and cell debris, lysis products, and the presence of heterotrophs were ignored while gravimetrically measuring the biomass. However, the characterization of the consortia showed dominance of autotrophic ntirifiers and the higher yield coefficients suggested better energy conversion efficiency and higher CO_2 fixation potential of the consortia. The mass production process yielded high active biomass for immobilization in the bioreactors.

Table.1. Optimum growth requirements of the nitrifying consortia

Consortium	Salinity (ppt)	pH	Temperature (°C)	Substrate Concentration (µg/ml)
AMOPCU-1 (Penaeids)	30	7.5	28	10
AMONPCU-1 (Non- penaeids)	15	8.5	28	10
NIOPCU-1 (Penaeids)	30	7	28	10
NIONPCU-1 (Non-penaeids)	15	7	28	10

Table 2. Alkalinity destroyed during mass production of consortia (AMOPCU-1 and AMONPCU-1)

Alkalinity Destruction of the Consortium AMOPCU-1 (Penaeids)			
pH Optimum	Total NH_4-N removed (g/L)	Total alkalinity destroyed (g)	Na_2CO_3 used to neutralize acid production (g)
7.5	1.242.0	7.452.0-9.1908	62.0
Alkalinity Destruction of the consortium AMONPCU-1 (Non-Penaeids)			
PH Optimum	Total NH_4-N removed (g/L)	Total alkalinity destroyed (g)	Na_2CO_3 used to neutralize acid production (g)
8.5	1.948.0	11.688-14.41532	78.0

Table 3. Growth and yield of ammonia and nitrite oxidizers during activation in 2L fermentor and on ammonia oxidizers on mass production in 200L fermentor

Nitrifying consortia	Biomass (g dry weigh/L) at the day of harvest (days)	μ_{max} (/hr)	Yield Coefficient (g NO_2-N/ g NH_4-N or g NO_3-N/ g NO_2N)
AMONPCU-1 2L	175.0 (25)	0.112	1.315
200L	125.3 (160)		0.667
AMOPCU-1 2L	159.2 (25)	0.105	2.08
200L	165.0 (260)		1.23
NIONPCU-1 2L	140.3 (25)	0.11	1.6
NIOPCU-1 2L	155.9 (40)	0.123	2.4

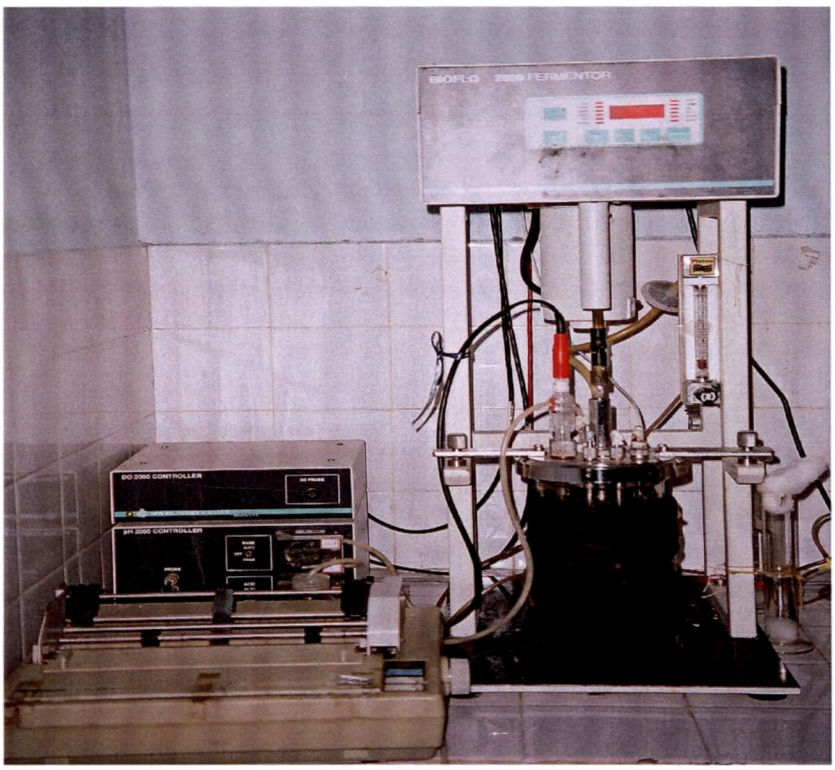


Fig.1. Amplification in 2L fermentor

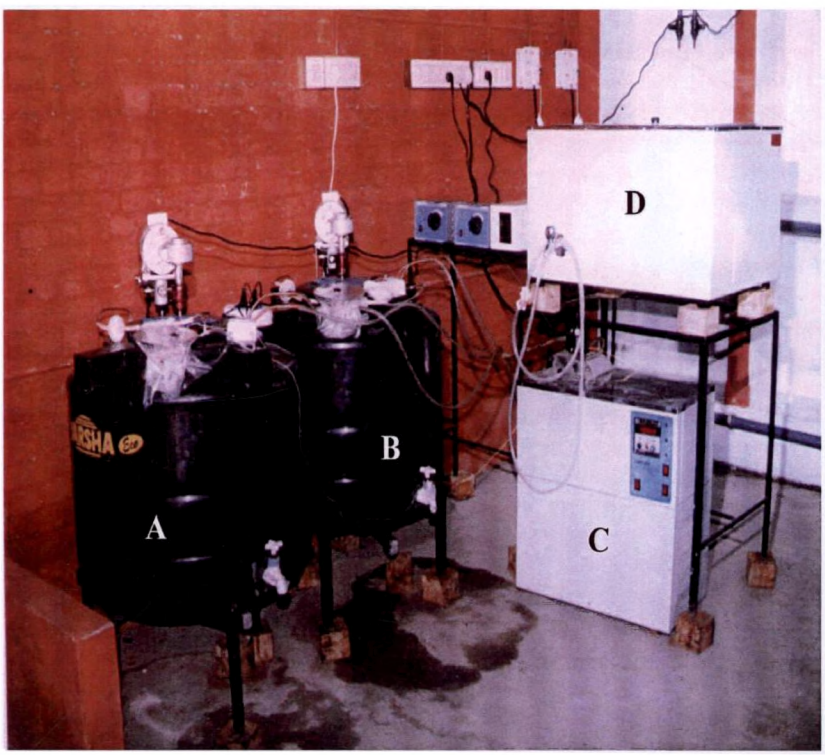


Fig.2. Nitrifying Bacterial Consortia Production Unit (NBCPU)
A and B-Fermentation vessel,C- Thermocirculator, D-Boiler

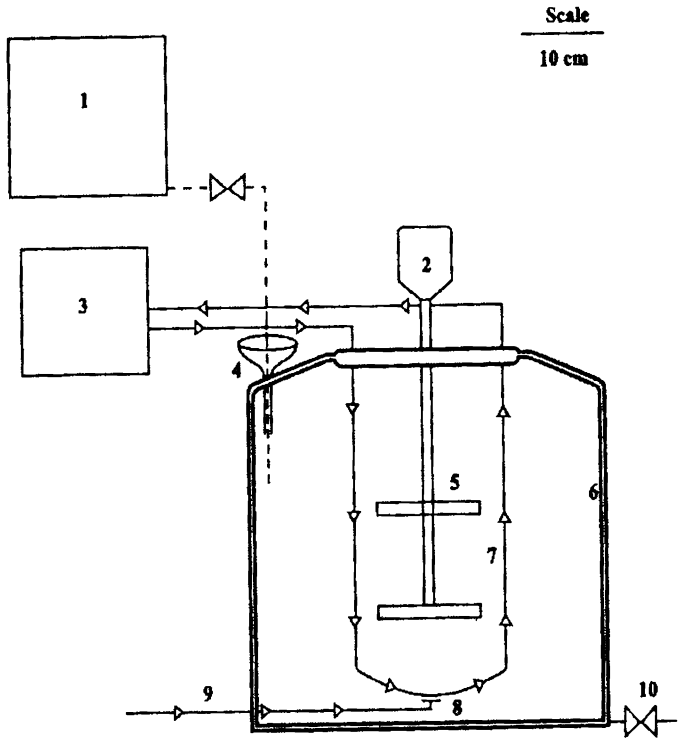


Fig. 3. Line diagram of the 200L NBCPU

1). Boiler 2). Motor 3). Thermo circulator 4). Addition/withdrawal port 5). Impeller 6). Polyethylene tank (200L) 7). Stainless steel heating coil 8). Air sparger 9). Sterile air liner 10). Drain pipe)

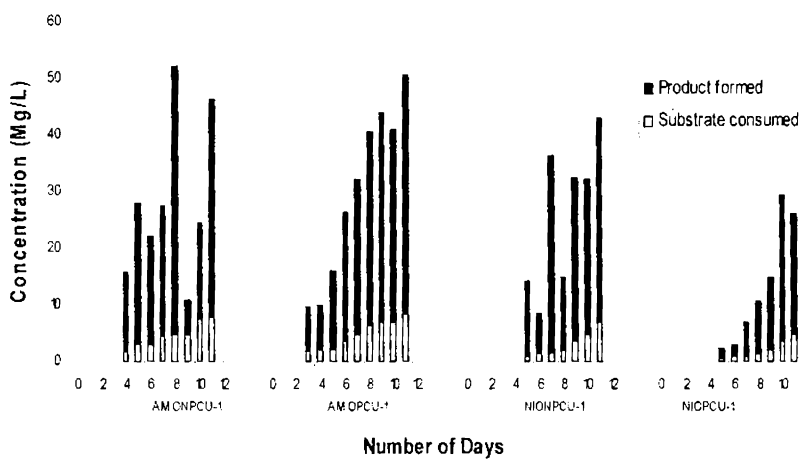


Fig.4. Pattern of substrate consumption and product formation during activation of stored (4°C) nitrifying consortia at room temperature.

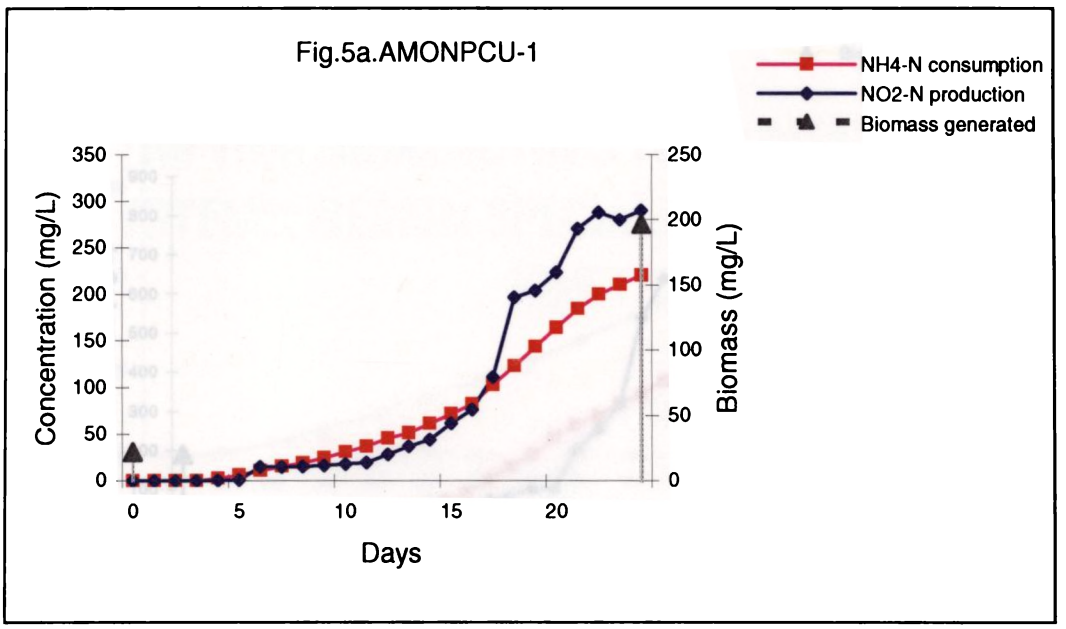


Fig.5a.AMONPCU-1
NH4-N consumption
NO2-N production
Biomass generated
Concentration (mg/L)
Biomass (mg/L)
Days
0
50
100
150
200
250
300
350
0
5
10
15
20

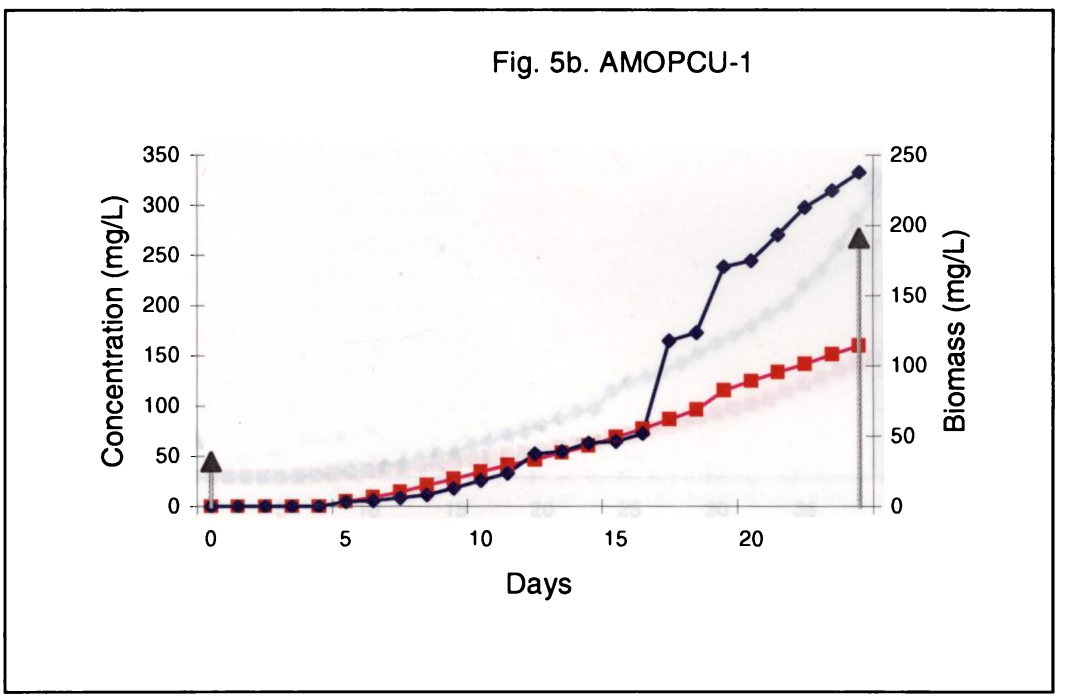
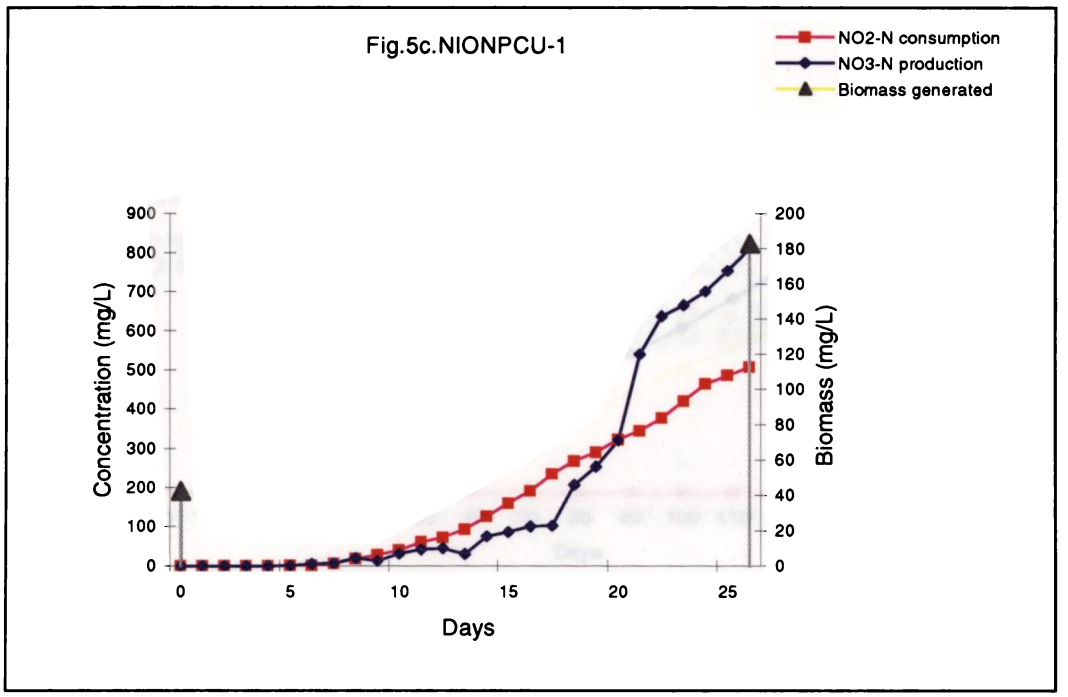


Fig. 5b. AMOPCU-1
Concentration (mg/L)
Biomass (mg/L)
Days
0
50
100
150
200
250
300
350
0
5
10
15
20



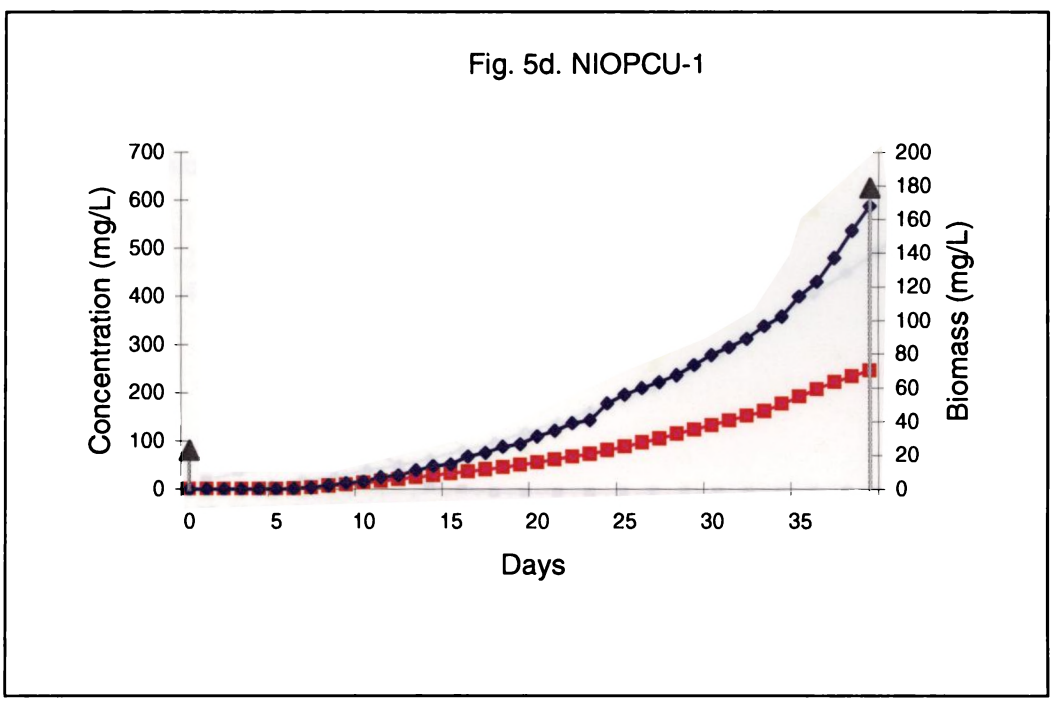
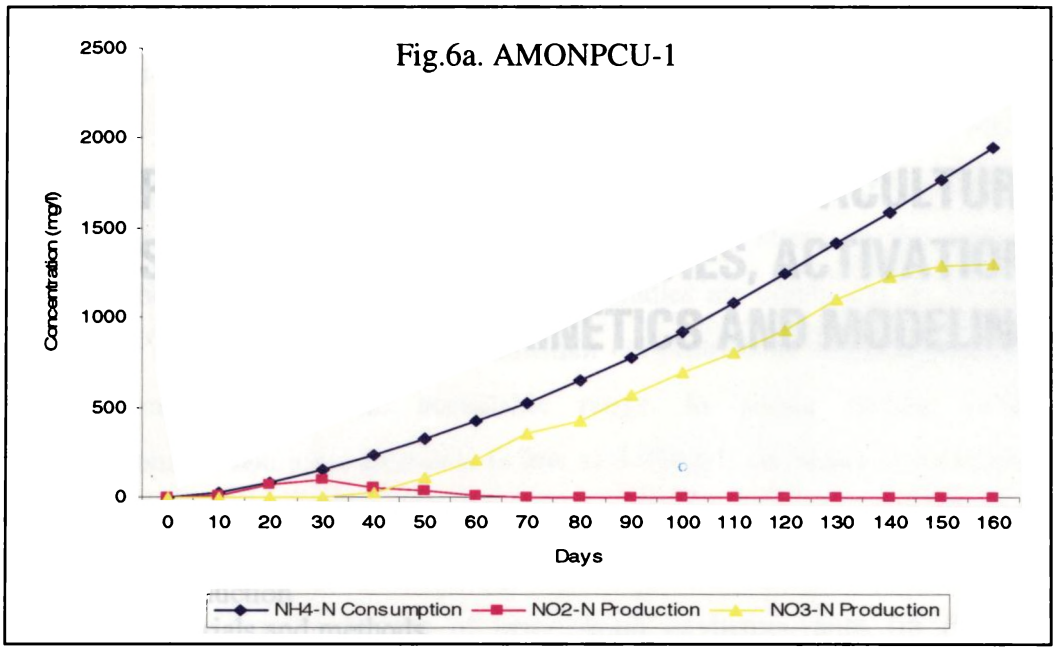


Fig.5. (a-d). Growth curves of nitrifying consortia in 2L fermentor



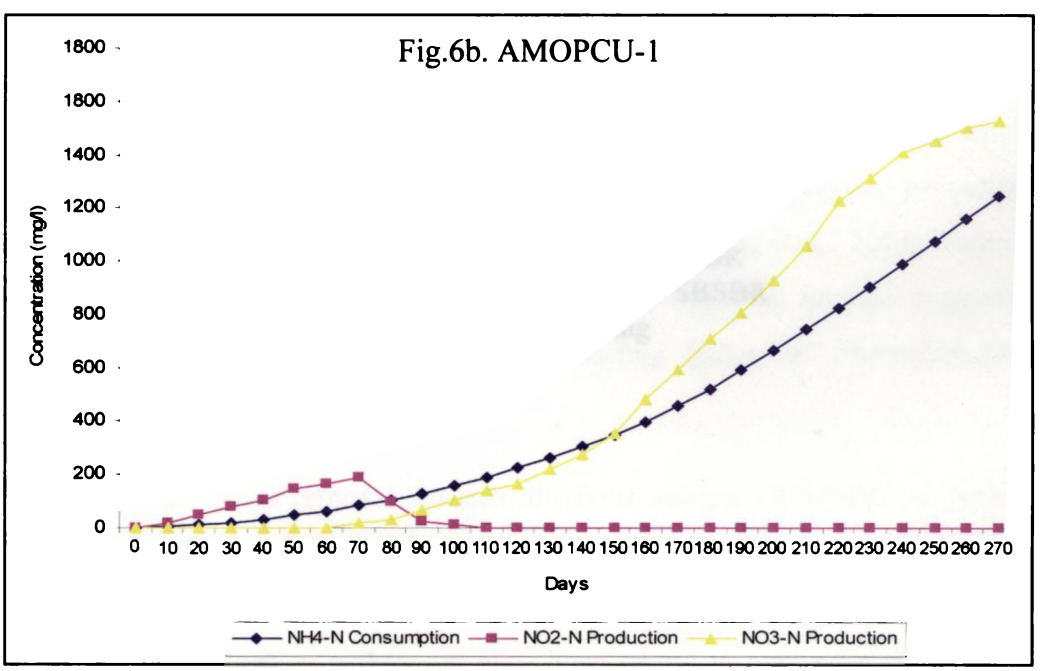


Fig.6 a-b. Growth of nitrifying bacterial consortia in 200L fermentor

Chapter 4

NITRIFYING BIOREACTORS FOR AQUACULTURE SYSTEMS: DESIGN SPECIALTIES, ACTIVATION, KINETICS AND MODELING

Contents

4.1. Introduction
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  4.2.3. Substrate kinetics and modeling of nitrification in the reactors
    4.2.3.4. Effect of organic carbon on nitrification
  4.2.4. Fluorescent In situ Hybridization (FISH) analysis of the mature biofilm
4.3. Results and Discussion
  4.3.1. Design specialties and activation of PBBR
  4.3.2. Design specialities and activation of SBSBR
  4.3.4. Nitrification kinetics and modeling

4.1 Introduction

Total ammonia nitrogen is the critical factor in aquaculture systems as it accumulates in the system as a byproduct of feed and animal excretion and it has direct effect on the health of aquatic animals. In a recirculating system, the wastes produced in the culture tanks must be removed at a sufficiently early to guarantee the required water quality in the system and to prevent stress on the cultured species (Wheaton et al., 1994). Most toxicity studies are conducted on juvenile animals for a relatively short time at constant concentrations while maintaining other parameters within an acceptable range. In young shrimp larvae, concentrations of non-ionic ammonia as low as 0.01mg/L can result in mortalities besides causing pathological disturbances as well as depressing growth rates (Ostrensky and Wasielesky Jr., 1995; Liu and Chen, 2004). Nitrite is also harmful to larvae as it causes reduction of hemolymph oxyhemocyanin (in *Penaeus monodon*) with concomitant increase in the partial pressure of oxygen (pO2) in haemolymph and reduced oxygen affinity (p50) (Cheng and Chen, 1995). However, it is less toxic than ammonia (Alcaraz et al., 1999) and, only under conditions of long term exposure the toxicity is found manifested (Wheaton et al., 1991) in the reared animals. Likewise, ammonia and nitrite toxicity in *Macrobrachium rosenbergii* adults and larvae have been investigated by various researchers (Chen and Lee, 1997; Cavalli et al., 2000; Wang et al., 2004; Naqvi et al., 2007). Nitrate is relatively harmless to the cultured aquatic organisms (Tomasso, 1994) and it has not been a limiting factor for *Macrobrachium* larviculture (Mallasen et al., 2004).

In biological ammonia removal systems nitrifying activity of bacteria suspended in seawater has been reported to be extremely low due to their slow growth rate and inhibition of nitrification by free ammonia and nitrite (Furukawa et al., 1993). However, immobilization techniques have been useful to overcome the situation (Sung-Koo et al., 2000) and accordingly, fixed film nitrification biofilters are commonly used for ammonia removal in Recirculation Aquaculture Systems (Wheaton et al., 1994; Seo et al., 2001; Shnel et al., 2002). In such installations

attached growth as biofilm offer several advantages over suspended culture based systems, such as handling convenience, increased process stability to shock loading and prevention of the bacterial population from being washed off (Fitch et al., 1998; Nogueira et al., 1998). Conventional fixed-film biofilters applied in aquaculture systems include: fluidized bed reactor, biological rotating contactor, trickling filter, submerged filter, and floating packed-bed reactor (Jewell and Cumming, 1990; Nijhof and Bonverdeur, 1990; Yang, Lin-sen and Shieh, 2001, Valenti and Daniels 2000; New 2002). New biofilter types being recently introduced to RAS include: moving bed reactor, three-phase fluidized filter, and hybrid biofilter. The significance of nitrifying biofilters in aquaculture has been reviewed well (Eding et al., 2006; Colt, 2006; Gutierrez-wing and Malone, 2006) and rating standards for these systems (Colt et al., 2006; Malone and Pfeiffer, 2006) are developed recently. Many of the submerged biofilters use crushed oyster shell, dolomite or coral (5 mm particles) as the filter media, but calcareous media contain an inexhaustible source of buffer material which slowly dissolves into the water. Therefore plastic filter media which have no buffering capacity are often used in biological filters (Seo et al., 2001; Sandu et al., 2002).

Biofilter selection influences capital and operating costs of recirculating aquaculture systems, their water quality, and even the consistency of water treatment (Summerfelt, 2006). A perfect biofilter would remove all of the ammonia entering the unit, produce no nitrite, and support dense microbial growth on an inexpensive support material that does not capture solids, require little or no water pressure or maintenance, and require a small footprint. However, no biofilter type commercially available has been found to meet all those objectives, nevertheless each one has its own advantages and limitations

A great deal of research has been conducted on the design and operation of major biofilters in aquaculture systems. In addition to the general reviews on characteristic advantages and simple engineering methods for commonly used biofilters (Wheaton et al., 1994; Timmons et al., 2001), researchers have provided

valuable information in terms of system design, operation and performance evaluations on fluidized bed reactors (Summerfelt et al., 1996; Sandu et al., 2002; Summerfelt, 2006), floating bead filters (Malone et al., 1998; Golz et al., 1999; Sastry et al., 1999; Malone and Beecher, 2000), trickling filters (Miller and Libey, 1986; Kamstra et al., 1998), and moving bed filters (Greiner and Timmons, 1998; Yossi Tal et al., 2003) for their applications in aquacultural systems. However, information on process mechanism and kinetics relative to nitrification biofilters applied to aquaculture systems is still insufficient. Simply employing data from traditional wastewater treatment processes to the design of aquaculture biofilters is not appropriate as nitrification conditions in aquaculture systems differs from domestic and industrial wastewater. Compared with domestic wastewater (Metcalf and Eddy Inc., 1991; Henze et al., 1997), aquaculture wastewater has a relatively low concentration of pollutants (Piedrahita, 2003). Aquaculture wastewater usually has a much lower ammonia substrate concentration, with total ammonia nitrogen (TAN) values of 20~50 mg/L in typical untreated domestic wastewater, 100~800 mg/L in septage systems (Tchobanoglous and Burton, 1991), and less than 1 and 3 mg/L of TAN for rainbow trout and catfish aquaculture systems, respectively (Wedemeyer, 2001).

Design criteria based on an average nitrification rate may be suitable for the design of biofilters operated at higher TAN concentrations where a zero order reaction could be applied. However, for low TAN concentrations, such as cold water systems (TAN < 1 mg/L), nitrification rate is affected greatly by TAN concentrations and consequently, the use of an average nitrification rate may not be appropriate for biofilter design. The resulting small variations in the design criteria because of the divergence from the use of an average nitrification rate could have a significant effect on the cost of large-scale filters, although the design of small biofilters may not be affected as much.

As biofilm nitrification process could be affected by various parameters, a kinetic study on major impact parameters is very important, especially for the effect of

substrate loadings, which is a key factor in categorizing different systems. Very limited work, however, has focused on the nitrification kinetics of aquaculture biofilters. Bovendeur et al. (1990) investigated nitrification kinetics of a trickling filter in a warm water system and found that the biofilter nitrification rate followed half-order kinetics for a TAN concentration of less than 2 mg/L, while zero-order kinetics was applied to a TAN concentration of 2 to 10 mg/L. A reduction of 0.015 g /m^2/d in nitrification per g /m^2/d of COD removal was also reported by the same authors. In a comparison study of nitrification performance between a micro-bead and a trickling filter in a tilapia culturing system, Greiner and Timmons (1998) reported that nitrification rates of both reactors increased linearly with influent TAN concentrations up to 2.5 mg/L. With a reactor series system, Zhu and Chen (1999, 2000, 2001, and 2002) conducted a series of experiments to quantify the effects of TAN concentration, organic matter, temperature, as well as hydraulic loadings (Reynolds number) on nitrification of submerged biofilters. Tseng and Wu (2004) studied the effects of temperature, ammonia, and suspended solids on biofilter ammonia removal efficiency and developed a regression model to provide operating guidelines for biofilter backwash frequency.

The nitrification rate in a biofilm is in equilibrium between substrate demand created by the growth of bacterial biomass and the rate of substrate supply determined by diffusion transport limitation (Rasmussen and Lewandowski, 1998). The substrate demand is determined by the factors that are related to the characteristics of nitrifiers such as the amount of nitrifier biomass, the specific growth rate and yield coefficient. The substrate supply is determined by the diffusion rate of essential nutrients (Chen et al., 2006). The nitrification rate is influenced by substrate concentration, dissolved oxygen (DO), mixing regime, pH, temperature, salinity and the level of organics (Chen et al., 2006). Techniques used for investigating biofilm reactor kinetics have included mainly two types of analyses: (1) local chemical and physical measurements inside the biofilm using microsensors (Lewandowski et al., 1991; de Beer et al., 1996; Stoodley et al.,

1997; Rasmussen and Lewandowski, 1998). and (2) chemical analysis of bulk solutions. In aquaculture systems, most studies were based on factors that can be measured in bulk water.

The concentration of TAN as the substrate of nitrification is the most important factor to consider in biofilter design and operation. In general, nitrification biofilters used in a RAS should be designed based on ammonia concentration (Wheaton et al., 1994). The minimum concentration that a biofilter can maintain and the relationship between nitrification rate and TAN concentration are very significant in aquaculture systems. The biofilters must be able to maintain a high quality of water with sufficiently low ammonia concentration and must process TAN at an adequate rate, at a given level, to prevent TAN accumulation.

This highest water quality that can be maintained in RAS, in terms of ammonia, is defined by a minimum substrate concentration that a biofilter can operate on a sustainable basis, S_{min}. The concept of a minimum substrate concentration required to support a steady state biofilm was proposed and proven by Rittmann and McCarty (1980) and Rittmann and Manem (1992). Rittmann and McCarty (1980) also mathematically defined the S_{min} for a biofilm as:

$$S_{min} = K_s \frac{b}{\mu_{max} - b} \qquad \text{.....(4.1)}$$

where, S_{min} is the minimum substrate concentration (mg/ L) and b is the specific bacterial decay rate/day.

Zhu and Chen (1999) evaluated the minimum TAN concentration for submerged nitrification biofilters in a reactor series system and the mean value of the minimum TAN concentration was found to be 0.07 ± 0.05 mg/ L at 27-28°C. Fortunately, the minimum TAN concentration was much lower than the toxic level for almost all the aquacultural species. This result provided the theoretical support for safely applying the fixed film nitrification process in RAS. A precise analysis of nitrification kinetics as a function of substrate concentration has to

consider the variation in substrate concentrations within the biofilm. However, it makes the estimations very complicated and for simplicity, attempts have been made to relate the nitrification rate directly to total ammonia concentration in the bulk liquid (Zhu and Chen, 1999). According to this approach, the ammonia concentration in the bulk liquid is treated as an apparent concentration that is implicitly related to the local concentration at a given point in the biofilm. The nitrification rate can be expressed by the maximum reaction rate with a correction term that reflects the effect of limiting substrate

$$R = R_{\max} \frac{S}{K_s + S} \qquad \text{.....(4.2)}$$

where, R: substrate oxidation rate (g/ m^2 /day; R_{max}: maximum substrate oxidation rate (g/ m^2 /day); S: limiting substrate concentration (mg/ L; K_S: half saturation constant (mg/ L).

The low concentration requirement in aquaculture systems determines that in most cases, TAN concentration is the rate-limiting factor of biological nitrification, however, due to the fact that aquaculture biofilters operate at lower TAN concentrations compared with those used in industry wastewater treatment, these equations can be simplified for aquaculture applications. At low ammonia substrate concentrations nitrification kinetics can be simplified into a first order reaction model (Zhu and Chen, 2001)

$$R = R_{\max} \frac{S - S_{\min}}{K_s + S - S_{\min}} \qquad \text{.....(4.3)}$$

The above relation shows that nitrification rates increase linearly with the increase of TAN substrate concentration and it has been confirmed with experimental aquaculture systems. Ester et al. (1994) studied the performance of three rotating biological contactor (RBC) systems used for RAS and observed first-order nitrification kinetics at low ammonia concentrations. Other researchers (Watanabe et al., 1980; Surampalli and Baumann, 1989) have also found that a first-order

reaction can be developed for RBC reactors at extremely low ammonia concentrations.

Dissolved oxygen is another important factor influencing nitrification kinetics. The theoretical oxygen requirements according to the nitrification stoichiometric equations are: 3.43 mg for oxidation of 1 mgNH_3–N and 1.14 mg for oxidation of 1 mg NO_2-N, although a slightly lower ratio of oxygen consumed to nitrogen oxidized in an experimental study was also reported (Wezernak and Gannon, 1967; Sharma and Ahlert, 1977). Based on pure culture studies the minimum range of DO concentration required to reliably achieve nitrification is between 0.6 and 3.4 mg/ L and DO of 2 mg/ L was suggested as the minimum oxygen level in aquaculture nitrification biofilters (Wheaton et al., 1994). However, limited oxygen conditions in a fixed film biofilter can be significantly different, as oxygen availability to nitrifying biofilm is subjected to diffusion limits. More than ammonia oxidizing bacteria, nitrite oxidizers are more sensitive to dissolved oxygen. Low DO concentrations can also cause nitrite accumulation in nitrification biofilters. Although there is no significant evidence to determine the optimum concentrations of bulk DO for nitrification reactors used in aquaculture systems, a DO greater than 2.3 mg/ L within the biofilm is necessary for the purpose of preventing nitrite accumulation which is a significantly negative factor for the growth of aquaculture species. The double Monod model should be used to incorporate the effect of DO into nitrification kinetics to account for simultaneous ammonia and oxygen limits (Stenstrom and Poduska, 1980; Beccari et al., 1992; Picioreanu et al., 1997; Hagopian and Riley, 1998).

$$R = R_{\max} \frac{S}{K_s + S} + \frac{C}{K_c + C} \qquad \text{..... (4.4)}$$

where, C is the concentration of DO in the reactor and K_C is the half saturation constant in terms of oxygen.

The nitrification rate may be significantly improved through increasing the turbulence as nutrient mass flux determines the efficiency of a fixed film biofilter.

Turbulence affects the thickness of the water film and subsequently the transfer resistance of substrate from bulk liquid into the biofilm. The transport of substrate in moving liquid is governed by molecular diffusion and advection (Lewandowski et al., 1992). Experimental studies have shown that the turbulence caused by air mixing had a significant impact on nitrification rate in the fixed film biofilters (Zhu and Chen, 2003). Hsu et al. (2001) examined the kinetic behaviors of nitrogen compounds in biofilm channeling under laminar and turbulent flow conditions and found that the flow velocity significantly influenced the nitrification and denitrification conversion rates.

The fecal material excreted by fish and uneaten feed is the source of organic carbon in aquaculture systems; both in dissolved and particulate forms. The most important impact of organics upon nitrification is due to the contribution of additional oxygen demand. With the addition of organic matter, fast-growing heterotrophic bacteria which use organic carbon as their energy source will out compete slow-growing nitrifying bacteria, resulting in a decrease in the nitrification rate. It was reported that heterotrophic bacteria have a maximum growth rate of five times and yields of two to three times that of autotrophic nitrifying bacteria (Grady and Lim, 1980). Okabe et al. (1996) discovered that a higher influent C/N ratio retarded accumulation of nitrifying bacteria and resulted in a considerably longer start-up period for nitrification. Satoh et al. (2000) found that an increase in substrate C/N ratio (using acetate) immediately induced the interspecies competition for oxygen between ammonia-oxidizing bacteria and heterotrophs at the outer region of a biofilm. As a result, the ammonia-oxidizing bacterial population was reduced and ammonia oxidation was restrained. Zhu and Chen (2001) in their experimental study proved that addition of sucrose carbon with a carbon/nitrogen ratio of C/N=1.0 or 2.0 reduced TAN removal rate nearly 70% as compared with a pure nitrification process (C/N=0). The potential of heterotrophic inhibitory impact on nitrifiers decreased with the increase in organic carbon concentration when C/N ratio above 1.0.

In aquaculture, two types of organic matter loading rates for trickling filters can be distinguished: 1) short-term peak loading rates (3–4 hr), often caused by diurnal variation in waste production due to the feeding strategies applied and 2) structural high organic matter loading rates due to, for example, low efficiency of the solids removal unit (Summerfelt et al., 2001), feed utilization differences among fish species (Heinsbroek, 1988), feed spill (Nijhof, 1994) or differences in feed composition (Cho et al., 1994). Therefore, effects of the COD/N ratio on biofilter nitrification capacity should be considered in the design of systems for aquaculture applications.

The other important factors affecting nitrification rates in biofilters are temperature, pH, alkalinity and salinity. The impact of temperature on the nitrification rate in a fixed film processes was greatly reduced due to influence on bacterial growth rate compared with its impact in a suspended culture process (Chen et al., 2006) The main reason for the reduction of temperature impacts on fixed film nitrification rate was due to the domination of the mass diffusion transport process in a fixed film biofilter. The DO saturation concentration in the bulk water decreases as temperature increases. Although the diffusion coefficient itself increases as temperature increases, the diffusion process limited the mass flux into the fixed biofilm and thus the DO can become a limiting factor within the biofilm. The high sensitivity values for the diffusion coefficient imply the importance of diffusion transport in a fixed film, which is much greater than the importance of the growth rate coefficient in mixed culture processes (Zhu and Chen, 2002). Flora et al. (1999) mentioned that nitrification rates may be improved by increasing bulk pH to high (alkaline) values, which results in optimum (neutral) pH within the biofilm. This is very significant for the operation of nitrification biofilters in aquaculture systems. Although a low pH is optimal in the culture tank to minimize the toxicity of the portion of unionized ammonia regardless of the optimal pH for the growth of aquaculture species, a pH higher than the optimum in the biofilter is desirable for the improvement of the biofilters nitrification efficiency (Chen et al., 2006). The impact of alkalinity on the

nitrification rate is also related to that of the pH. Chen et al. (1989) showed that the rate of nitrification would be reduced when alkalinity was below 40 g/m^3.

Relatively limited information is available concerning the effect of salinity on nitrification kinetics (Chen et al., 2006). Nijhof and Bovendeur (1990) on comparing the nitrification characteristics of salt water RAS with that of fresh water systems observed that the maximum nitrification capacity in the salt water systems was considerably lower than in fresh water systems. There is an agreement among researchers and between laboratory research and commercial applications in that salt water systems need a much longer start-up period in which nitrite accumulates. The cause for these problems remains unknown, and has been attributed to a number of factors, such as high organic loadings (Van den Akker et al., 2003), or changes in salt concentration and operational parameters (Svobodova et al., 2005). Gutierrez-Wing and Malone (2006) suspected that they are being controlled either by the lack of proper genetic seed or by unrecognized factors influencing the establishment of marine nitrifying populations. In such cases biofilters have exhibited low performance in some cases, besides demanding too long a start- up period imposing operational difficulties (Sung-Koo et al., 2000). Therefore, particularly in marine environments, an acclimation period is necessary before a biofilter begins to operate properly (Manthe and Malone, 1987). The acclimation can be achieved by initial light loading with organism or ammonia and nitrite addition

Therefore, nitrifying bioreactors with short start-up time and easiness to integrate to the existing hatchery designs without modifications need to be developed. The sizing of biofilters in aquaculture systems are based on feed input and biomass load into the system. However, it is not always practical to change the size of the biofliter based on the load into the system. Instead increasing the cell density of the consortia used for activation will be a pragmatic approach. Based on this, the two types of reactors developed, Packed Bed Bioreactors (PBBR) and Stringed Bed Suspended Bioreactors (SBSBR), were activated with indigenous nitrifying

bacterial consortia and the activation kinetics was studied. The nitrification kinetics of activated PBBR in laboratory experimental systems was evaluated in terms of the effects of flow rate on substrate removal. The nitrification kinetics of SBSBR was evaluated under different substrate concentrations and C/N ratios. The Monod model was applied to the experimental data on substrate utilization to derive the TAN removal rates and the predicted values were compared with the experimental values by fitting a regression model.

4.2 Materials and methods

4.2.1 Activation of the packed bed bioreactor (PBBR)

4.2.1.1 Fabrication of PBBR

Cross sectional view of the nitrifying bioreactors (ammonia oxidizing and nitrite oxidizing) connected serially is given in Fig. 1. Both the reactors have the same configuration consisting of a shell made of fiberglass with a base of 30 cm^2 and an overall height of 45 cm. Nine PVC pipes (air lift pumps), each of 30 cm long and 2 cm diameter, fixed at 10 cm equidistance is fitted to a perforated base plate made of Perspex, and is positioned at the base of the reactor. When air gets passed through the air lift pump, the 10 cm^3 area surrounding each airlift pump, filled with the support medium, acts as an aeration cell. The base plate is elevated by 5 cm from the bottom supported by 5 cm long PVC pipes having 3 cm diameter. An inlet pipe is fixed at a water discharge height of 35 cm up from the base of the reactor. The outlet pipe, which emerges from the base of the reactor, bends upward at water discharge height of 35 cm from the base to the next reactor.

A 5mm of diameter and 0.785 cm^2 surface area beads with spikes on their surface were used during the study. Polystyrene (PS) and low density polyethylene (LDPE) were selected as suitable support materials for immobilizing ammonia and nitrite oxidizing consortia respectively, on the basis of percentage consumption of NH_4-N/NO_2-N and production of NO_2-N/NO_3-N by the immobilized nitrifiers on the beads, cost of the raw material and easiness to mould into beads (Achuthan, 2000). The beads were immersed in 0.1 N HCl for 3 hr, washed with 10 % Extran (mild phosphate free detergent) (Enviroeuip, Sydney,

Australia), rinsed with tap water followed by distilled water and air-dried. The reactor 1 (ammonia oxidizing) was filled with 60,000 PS beads and the reactor 2 (nitrite oxidizing) with the same number of LDPE. The fully assembled activated reactors (Fig. 2) can be readily integrated into the hatchery systems. The media and filter characteristics of the reactor are described in Table 1.

4.2.1.2 Activation with nitrifying bacterial consortia

The packed bed reactors were activated with ammonia oxidizing non-penaeid culture-1 (AMONPCU-1) and nitrite oxidizing non-penaeid culture-1 (NIONPCU-1) at 15 ppt and with ammonia oxidizing penaeid culture-1 (AMOPCU-1) and nitrite oxidizing penaeid culture-1 (NIOPCU-1 at 30 ppt (Achuthan et al., 2006) under optimized growth and culture conditions. This consisted of simple seawater based media at 15/30 ppt supplemented with 10 mg/L substrate $(NH_4)_2$ SO_4 for ammonia oxidizers and $NaNO_2$ for nitrite oxidizers), 2 mg/L KH_2PO_4 at an optimum temperature of 28° C with pH 8.5 for ammonia and 7.5 for nitrite oxidizers. After harvesting, the cultures were maintained at 4°C with periodic addition of the substrate ($(NH_4)_2$ SO_4/ $NaNO_2$) and adjustment of pH (using 1% Na_2CO_3) to the optimum. The consortia were further mass produced as earlier (Singh et al., 2007) to facilitate their immobilization in the reactors.

The ammonia oxidizing and the nitrite oxidizing consortia (20 L each) were introduced into the reactor 1 and 2 respectively and airlift pumps operated by supplying 1 L/min to effect adequate circulation of the culture through the beads and to assure supply of O_2 and CO_2 for activation. Optimum culture conditions were maintained in both the reactors during the activation period. The substrate concentrations (NH_4-N/NO_2-N) in both the reactors were made up to 10 mg/L daily by the addition of aqueous ammonium sulphate or sodium nitrite. Evaporation loss was made up by adding distilled water daily. The activation was continued for a week. The activation experiments were repeated two times as above.

When the reactors were in the activation mode, substrate/product levels were determined daily by estimating ammonia (TAN) (Solarzano, 1969), nitrite (NO_2-N) (Bendschneider and Robinson, 1952) and nitrate (NO_3-N) (Strickland and Parsons, 1972). The nitrifying biomass was determined gravimetrically by passing 10 mL bacterial suspension from the reactors through pre-weighed cellulose acetate syringe filters of 0.22 µm porosity with a diameter of 13 mm.

The simple correlation coefficient analysis between removal of ammonia and nitrite and the biomass in suspension during the activation mode was carried out.

4.2.2 Activation of stringed bed suspended bioreactor (SBSBR)

4.2.2.1 Configuration of SBSBR

The Stringed Bed Suspended Bioreactor (SBSBR) was developed for instant nitrification in larval rearing tanks. The reactor has four components (1) an outer shell of 10 cm^3 with conical bottom; (2) inner cartridge comprising a solid frame work and beads on strings with filter plates both at its top and bottom; (3) an airlift pump at the centre of the filter plates, and (4) a black lid at top with perforations. Based on a previous study by Achuthan (2000), polystyrene and low density polyethylene beads with 5mm diameter and a surface area of 0.785 cm^2 with spikes on the surface had been selected as the substrata for immobilizing ammonia and nitrite oxidizing consortia respectively. On full assembly (Fig. 3), the cartridge with beads was inserted into the outer shell and the black lid was placed at top. The reactor provided an overall surface area of 684 cm^2 to support the nitrifying biofilm.

4.2.2.2 Activation with nitrifying bacterial consortia

The reactors were activated with AMOPCU-1 and AMONPCU-1 and two nitrite oxidizing consortia such as NIOPCU-1 and NIONPCU-1 (Achuthan et al., 2006). The consortia were acclimated to room temperature (27±0.5°C) in 250 mL conical flasks on a rotary shaker for 7 days, from refrigeration (4 °C) and subsequently amplified in a 2 L baby fermentor (New Brunswick, USA) for one month under optimum pH (8.5 and 7.5), temperature (28°C) for ammonia and nitrite oxidizers

respectively. This was used as the inoculum for mass production in an indigenous 200 L nitrifying bacterial consortia production unit (Singh et al., 2007) under the optimum conditions as above. The reactors for penaeid hatchery systems were activated maintaining salinity optima of 25-30 ppt, and non-penaeid systems under salinity 10-15 ppt.

For activation, lid of the reactor was removed; void volume filled with an aliquot of 750 ml respective nitrifying bacterial consortium and clamped in place of a thermostatically controlled serological water-bath which served as the activator. The consortia were circulated through the cartridge by operating the airlift pump at a rate of 1 L/ min. For avoiding the escape of aerosols during the operation, top of the reactor was covered with 'aerosol arrestor', a cup shaped device made of perspex having a central hole for extending aeration tubing to the air lift pump. The media composition and culture conditions were the same as above. NH_4^+- N and NO_2^--N were monitored daily and adjusted to the optimum of 10 mg/L (Achuthan et al., 2006). Change in concentrations of the substrates (NH_4^+-N/NO_2^--N), and the products (NO_2^--N/NO_3-N) and disappearance of the nitrifying bacterial biomass from bulk volume (gravimetric determination) were considered to assess the extend of activation of the reactors.

4.2.3 Substrate kinetics and modeling of nitrification in the reactors

The kinetics of substrate utilization in the activated reactors was carried out under different conditions. To activate the reactors with minimal inocula, total number of cells in the consortia was quantified by epifluorescence microscopy

4.2.3.1 Determination of the cell count

The consortia were sonicated at 125 W for 4 min using an ultrasonicator (Vibra cell, Sonics, USA). Sonicated samples were stained with DAPI (5µg/mL) and filtered through 0.2µm Iregalan black stained nucleopore polycarbonate filters (Millipore GTBP011300). To get uniform cell distribution, base of the filtration apparatus was wetted prior to placement of the wet polycarbonate membrane. After filtration, nucleopore filters were mounted immediately on a slide using

non-fluorescent immersion oil and observed under Olympus CX-41 epifluorescent microscope. An eyepiece of known area was used during enumeration.

Calculation

$$\text{Bacterial abundance (cells/L)} = \frac{C_f \times R \times 1}{F_s} \quad \text{.....(4.5)}$$

C_f = mean number of cells/field

R = Active area of the filter/ area of field counted

F_s = Volume of fluid filtered (L)

Subsequent to enumeration aliquots of 50 mL were added to the reactors (both PBBR and SBSBR) to supply a cell density of 3-4 $x10^5$ cells/mL.

4.2.3.2 Effect of flow rate on nitrification

The effect of different flow rates on substrate removal kinetics of the activated packed bed reactor system (PBBR) connected in series (3 each in parallel series) was studied at the shrimp maturation system operated at West Coast Hatchery, Alapuzha, Kerala, India. The reactors were activated with AMOPCU-1 and NIOPCU-1 at 30 ppt till steady state was attained (TAN removal rate equaled the rate of addition, Zhu and Chen (2001)). Instead of synthetic feed solution, a simple sea water based medium with NH_4Cl as substrate was used for the experiments due to two reasons, 1) the consortia used for activation was enriched in sea water based media and 2) to simulate the hatchery conditions. The feed solution was prepared in 30 ppt sea water supplemented with 1000mg/L NH_4Cl to get a substrate concentration of 2 mg/L. The feed solution (2000L) was continuously pumped into an overhead tank (100 L) of the reactor and from where flowed down by gravitation through the series of reactors at the flow rate of 200 L/hr and the effluent were collected in an effluent collection tank (100 L). Pumping was controlled by an automated water level controller (V-guard, Kerala, India) fitted inside the overhead tank and a regulator valve connected to the overhead tank maintained the flow rate. The average temperature was 28±2°C. Concentrations of TAN, NO_2-N and NO_3-N were measured at different sampling points- overhead head tank, outlets of the six reactors and collection tank. The

experiment was repeated three times. The flow rates were then changed to 400 L/hr and 600L/h at the same substrate concentration of 2 mg/L and the experiment was repeated for each flow rate as above. The experiments were repeated three times at all the flow rates.

From the measured TAN concentrations the average TAN removal rates of the reactor system at each flow rate was calculated as follows (Zhu and Chen, 2001):

$$R_i = Q(S_0 - S_1)A_i \qquad \text{.....(4.6)}$$

where *Ri* is the TAN removal rate of reactor *i* (mg/ m^2 /d); *Q*, the flow rate through the series (L/day); *Si*, the TAN concentration of the outflow from reactor series (mg/L) (S_0 is the TAN concentration of feed solution); *Ai*, the total biofilm surface area of all the reactors in series (m^2).

Significance in the rate of removal of TAN and NO_2-N and production of NO_3-N in the reactors was analyzed by one-way ANOVA.

4.2.3.3 Effect of substrate concentration on nitrification

The effect of TAN concentration on nitrification in the Stringed Bed Suspended Bioreactors was evaluated in an experimental system maintained in triplicate at salinities 15 and 30 ppt. Before starting with the experiment, the optimum flow rate for SBSBR was determined from the maximum turn over of the feed solution at different air flow rates of 1, 2 and 3L/min and for concurrent results the experiment was repeated six times and based on the results water flow rate was fixed 3L/min. The reactors were activated with AMOPCU-1, NIOPCU-1, AMONPCU-1 and NION-PCU-1 (50 mL each) till steady state was attained (TAN removal rate equaled the rate of addition, Zhu and Chen (2001)). The feed solution was prepared with 50L seawater (15 and 30 ppt) having substrate (NH_4-Cl) concentration of 1 mg/L in 100L FRP tank. The reactors were immersed in the feed solution and circulated through the cartridge by operating the airlift pump at a flow rate of 3 L/ min at 28±2°C. The TAN, NO_2-N and NO_3-N were estimated at intervals of 2hr for 12 hr. The experiments were performed as above

at concentrations of 2, 3 and 4 mg/L. In between each treatment the reactors were activated at the respective concentration before starting with the measurements.

The TAN removal rates at the end of each experiment were estimated following Eq. 4.6. The relationship between TAN and TAN removal rates was delineated from the experimental data following Monod kinetics (Eq. 4.3). The R_{max} was calculated by least square method of the experimental data. Ks for ammonia was taken from literature as 1 mg/L (Henze et al., 1997) as it is the commonly accepted value. A minimum TAN concentration of 0.07 mg/L (Zhu and Chen, 1999) was used in the model and TAN removal rates at each TAN concentration were predicted by the simplified model:

$$R = R_{max} \frac{S - 0.07}{K_s + S - 0.07} \quad \text{.....(4.7)}$$

The linear regression between estimated and predicted TAN removal rates was performed. The difference between percent TAN removals between different TAN concentrations over time was tested by two way ANOVA.

4.2.3.4 Effect of organic carbon on nitrification

The impact of loading of organic carbon on nitrification in the Stringed Bed Suspended Bioreactors was evaluated in a short duration experiment. The experimental systems were maintained in triplicate at salinities of 15 and 30 ppt. The reactors were activated with AMOPCU-1, NIOPCU-1, AMONPCU-1 and NIONPCU-1 (50 mL each) till steady state was attained (TAN removal rate equaled the rate of addition, Zhu and Chen (2001)). The feed solution contained NH_4Cl to give a substrate concentration of 2 mg/L in all C/N ratios. Sucrose ($C_{12}H_{22}O_{11}$) was used as a carbon source to control the weight ratio of carbon to nitrogen (2375mg sucrose gives 1mg C). The experiment was started with C/N=0, followed by C/N=2 and C/N=4. The reactors were filled with 0.75L of feed solution each and maintained in a temperature controlled chamber at 28°C and the TAN, NO_2-N and NO_3-N were estimated after 1 hr. The measurements were repeated with fresh feed solution three times. Prior to the commencement of the

experiment with the next C/N ratio the reactors were activated at the respective concentrations. The percent TAN removal was calculated for each C/N ratio and the difference analyzed by one way ANOVA.

4.2.4 Fluorescent in situ hybridization (FISH) analysis of the mature biofilm

The diversity of nitrifiers present in mature biofilms which have undergone a prolonged period of operation was analyzed by FISH. Biofilm was collected from beads of a PBBR, which was under operation continuously for 6 months at the National Centre for Aquatic Animal Health Laboratory, Kochi, India. These reactors were the ones activated with the nitrifying bacterial consortia AMONPCU-1 and NIONPCU-1 and were under operation at salinity of 15-18 ppt under a TAN concentration in the range of 1-2 mg/L. Altogether, 25 beads were taken from the reactors and the biofilm was dislodged using a cyclomixer. The biofilm samples were centrifuged and fixed for fluorescent in situ hybridization (FISH) analysis. The FISH analysis of the biofilm was carried out using a Universal bacterial probe and seven nitrifiers specific probes as described earlier. For observing the morphology of the cells, the biofilm was also stained with 1 µL (5 mg/L) DAPI.

4.3 Results and discussion

4.3.1 Design specialties and activation of PBBR

Proper selection and sizing of biofilters are critical to the technical and economic viability of RAS (Malone and Pfeiffer, 2006). The nitrifying PBBRs are packed with plastic media having a specific surface area of $205 m^2/m^3$ (Table1). This is comparable to the specific surface areas of plastic substratum found suitable in trickling filters used in aquaculture (Kamstra et al., 1998). The plastic beads with spikes on the surface provide high void ratios that avoid clogging (Eding et al., 2006) and increase the aeration within the system. The flow of water in the reactor system was maintained by gravitational force and energy required for the reactor operation could be restricted to pumping water to the reservoir tank and to operate an air pump to effect aeration. In case of incomplete nitrification after a single circulation, there is provision to recirculate it through the treatment system

over and again. However, this may be overcome by increasing the biomass of the nitrifying consortia used for activation of the reactors or by enhancing the hydraulic retention time. Further, the system can be upgraded with different types of filters for removal of particulate matter and UV disinfection equipment for elimination of pathogens which may enter the system accidentally. Another advantage of the reactor is that it is interchangeable between prawn (salinity 15ppt) and shrimp (salinity 30 ppt) larval rearing systems by replacing the nitrifying bacterial consortia (Achuthan et al., 2006). A modification of the system can be used for shrimp maturation facility too as recirculation is one of its prime requirement on water quality and biosecurity perspectives.

Activation kinetics of PBBR during the period of immobilization of the ammonia and nitrite oxidizing bacterial consortia (Fig. 3) showed that in all the reactors nitrification could be established within 24 hr of initiation of the process. There was progressive reduction in the suspended biomass and increase in NO_2-N and NO_3 - N respectively. The system was monitored for 7 days, during which there was a reduction of more than 90% of the bacterial biomass from the activation medium with 78% TAN and 75.3 % NO_2-N removal for the systems activated with AMONPCU-1 and NIONPCU-1 with a negative correlation between the percentage removal of TAN ($r = -0.96$, $P<0.01$), NO_2-N ($r = -0.93$, $P<0.01$) and suspended biomass in the reactor. The TAN and NO_2-N removals were 80.7% and 76.30% for reactors activated with AMOPCU-1 and NIOPCU-1 with a negative correlation between the percentage removal of TAN ($r = -0.93$, $P<0.01$), NO_2-N ($r = -0.90$, $P<0.01$). The average ammonia and nitrite removal rates in terms active bead surface area at the end of the activation period were 0.47 g TAN/m^2 /day and 0.45 g NO_2-N/m^2 /day for the systems activated with AMONPCU-1 and NIONPCU-1, whereas those for the systems activated with AMOPCU-1 and NIOPCU-1 were 0.48 g TAN/m^2 /day and 0.37 g NO_2-N/m^2 /day. Due to the low concentrations of nutrients in the aquaculture systems (Piedrahita, 2003) bacterial yield is also low and therefore activated bioreactors (Valenti and Daniels, 2000) with high attached bacterial density is essentially

required for optimal performance. During activation, nitrification could be established in both the reactors within 24 hr of initiation. Replacement of the activation fluid with fresh seawater having the same salinity did not affect the rate of nitrification suggesting the formation of active biofilms by nitrifying bacterial consortia. Wherever such activations had not been reported, in some cases it took 2-3 months for the establishment of nitrification in marine systems (Manthe and Malone 1987) and 2-3 weeks in freshwater (Masser et al., 1999).

4.3.2 Design specialties and activation of SBSBR

The filter and media characteristics of SBSBR are given in Table 2. The specific surface area of the media was 150 m^2/m^3. The SBSBRs described here (Fig. 4) are designed for setting up nitrification in shrimp/prawn larval rearing tanks during the operation. The technology is relatively user friendly in the sense that they can easily be lifted out of water during disinfection and also can be shifted from one rearing tank to another. Loss of beads encountered in many biofilter systems (Timmons et al., 2006) are not experienced in the case of SBSBRs as the beads are stringed together within the reactors. Moreover, the operational costs of the reactors are minimal and no energy costs are added up to the overall production cost as the aeration system already available in the hatchery are used for operating the airlift pumps. SBSBRs can also find application during the live transportation of spawners as their survival is diminished mainly due to the ammonia produced by them (Babu and Marian, 1998). The reactors are sufficiently smaller enough to be incorporated in to the transportation containers.

Activation kinetics of SBSBRs is presented in Fig. 5. Nitrification could be established in all bioreactors within 24 hr of initiation of the process and there was progressive reduction in the suspended biomass and increase in NO_2-N and NO_3-N respectively. By the 7^{th} day, more than 90% of the bacterial biomass had disappeared from the activation medium with a removal of 80.70 and 78.10 % ammonia and 76.20 and 75.30 % nitrite respectively in the reactors meant for non-penaeid and penaeid larval production systems. The average ammonia and

nitrite removal rates at the end of the activation period were 0.88 and 0.86 g TAN/m^2/day and 0.84 and 0.83 g TNN /m^2/day, respectively.

In terms of TAN removal rates per unit of medium surface area, Westerman et al. (1993) reported 0.25g TAN removal/ m^2/day for rotating biological contactors (RBC) and 0.1-0.15g TAN/m^2/day for up-flow sand bead filters. For a trickling filter, Van Rijn and Rivera (1990) reported a removal rate of 0.43 g TAN/m^2/day. The average TAN removal rates (g TAN/ m^2/ day) reported for frequently used biofilters in aquaculture systems are; rotating biological contactors 0.19-0.79, trickling filters 0.24-0.64, bead filters 0.30-0.60 and fluidized sand filter 0.24 (Crab et al., 2007). The average TAN and TNN removal rates of SBSBRs during the activation period were higher than the reported values.

4.3.3 Nitrification kinetics and modeling

4.3.3.1 Determination of the cell count

The cell counts of the bacterial consortia used for kinetic studies were in the range of 3-4x 10^5 cells/mL. Fluorescing images less that 0.2μm in diameter were disregarded during the enumeration. Quantitative determination of the nitrifying bacterial consortia having larger aggregates, biofilm and flocs require reliable methods for efficient, nondestructive de-aggregation of the cells. Ultrasonication has been reported to be a suitable technique for de-aggregation of bacterial flocs (Salhani and Deffur, 1998), which was followed here.

4.3.3.2 Effect of flow rate on nitrification

The flow rate has a significant effect on nitrification as it influences the turbulent mixing and thus mass transfer of substrate and nutrients into the biofilm. The concentrations of average TAN, NO_2-N and NO_3-N at different flow rates in the reactor series (Fig 6a -6c) showed no significant difference between the series, but there was significant difference as the feed solution passed from the overhead tank down to the series of reactors. At 600 L/hr the average initial and final TAN across the reactor series were 2.20 and 1.72 mg/L ($P<0.05$), NO_2-N, 0.164 and 0.22 mg/L (not significant) and NO_3-N

were1.57 and 2.0 mg/L ($P<0.001$) respectively. At the flow rate 400 L/hr the values were TAN, 2.31 and 1.71 mg/L ($P<0.001$), NO_2-N, 0.07 and 0.21 mg/L ($P<0.01$) and NO_3-N, 2.9 and 3.8 mg/L ($P<0.001$) respectively. The average initial and final TAN, NO_2-N, and NO_3-N at the flow rate of 200L/hr were 2 and 1.04 mg/L ($P<0.001$); 0.12 and 0.55 mg/L ($P<0.05$), and 2.60 and 4.20 mg/L ($P<0.05$) respectively. The NO_2-N was always maintained low, an observation similar to the substrate removal kinetics of the consortia during mass production. The average volumetric TAN removal rates increased with flow rates from 33.04 (200 L/hr) to 49.43 (600 L/hr) gTAN/m^3/day (Table 3) whereas the percent TAN removal decreased with increased flow rates. The increase in the average TAN removal rates is due to the increased turbulence and subsequent mass transfer of substrate into the biofilms during the high flow rate. The decrease in the percent TAN removal rates with increase in the flow rate coincides with the decreasing HRT. Stoodley et al. (1997) investigated the relationship between local mass transfer coefficients and fluid velocity in heterogeneous biofilms using microelectrodes and confocal scanning laser microscopy and found that the effects of biofilm heterogeneity on mass transport were strongly dependant upon the average flow velocity. de Beer et al. (1996) measured DO concentration profiles on heterogeneous biofilm and found that the thickness of the mass transfer boundary layer on the film decreased exponentially with increasing flow velocity. Zhu and Chen (2001) investigated the relationship between total ammonia nitrogen removal rate and the Reynolds number (Re) in a steady-state nitrification fixed biofilm and observed that the Reynolds number of the flow had a significant impact on the ammonia removal rate demonstrating that the hydraulic condition of the biofilm surface was a major factor affecting TAN removal rate. In another study by Zhu and Chen (2003) it was shown that the turbulence caused by air mixing had a significant impact on nitrification rate in the fixed film biofilters suggesting that increasing turbulent air flow could be an effective method to improve the nitrification efficiency of fixed film biofilters.

4.3.3.3 Effect of substrate concentration on nitrification kinetics, and modeling

The concentration of TAN, NO_2-N and NO_3-N in the feed solution on circulation through the SBSBR showed that at all substrate concentrations and salinities of both 15 and 30 ppt the nitrification progressed with time (Fig. 7a-7d and 8a-8d respectively) whereas in the control there was no nitrification. At 1 mg/L TAN, the concentration decreased to 0.18 and 0.17 mg/L respectively in reactors activated at 15 and 30 ppt, whereas TAN concentrations at substrate loadings of 2,3, and 4 mg/L at 15 and 30 ppt salinity were 0.95 and 0.92 mg/L; 1.68 and 1.56 mg/L and 2.26 and 2.7 mg/L respectively. This clearly shows that salinity does not have a significant effect on the nitrification capacity of bioreactor under the experimental conditions. The percentage TAN removal was highest at 1 mg/L TAN ($P<0.001$) compared to higher concentrations at 15 and 30 ppt (Fig. 9a and 9b) and increased with time. Two way ANOVA results showed that there was significant difference in the TAN removal rates over time at different concentrations ($P<0.01$), but the difference between salinities of 15 and 30 ppt were significant only at 5% level.

The average TAN removal rates at TAN landings of 1, 2, 3 and 4 mg/L were 110.12±9.04, 148.61± 17.97 191.86± 2.67 218.78± 14.75 gTAN/m^2/day at 15 ppt whereas the values were 104.60±2.45, 88.02±18.13, 173.06±12.70, 223.05±7.25 gTAN/m^2/day at 30 ppt. TAN removal rates were predicted following Monod model and the experimental and predicted TAN removal rates at both salinities showed a good fit ($P<0.001$) (Fig. 10a-10b) showing that substrate removal rates followed first order Monod kinetics and TAN removal rates increase linearly with the increase of TAN substrate concentration (Zhu and Chen, 2001). In trickling filters also the rate of removal of substrates from the recirculating water is determined by their diffusion rates into the biofilm (Eding et al., 2006). Substrates first diffuse from the bulk liquid into the biofilm through a stagnant water layer and then into the biofilm. Once in the biofilm, the substrate is consumed by bacteria in accordance with the Monod kinetics. Oxygen and TAN

are usually limited in aquaculture systems, however in the present systems integrated with SBSBR aeration was provided to the optimum that O_2 levels were always maintained at 5-6 mg/L.

There nitrification rates at both salinities were higher compared to the already observed values and there was no delay in the initiation of nitrification. It was found that for seawater trickling filter biofilms, a slower start up to 'full-grown biofilm stage' and a lower TAN removal rate was observed when compared with freshwater biofilms (Nijhof and Bovendeur, 1990). A considerably longer start-up period (170 days) was needed for seawater trickling filter biofilm to reach the 'full-grown stage' when compared to a freshwater biofilm (130days). Nitrite removal rate in seawater is significantly slower to develop than in freshwater. A considerably larger nitrite accumulation was observed when compared to freshwater during the first months of the startup phase due to the slow development of the nitrite oxidation capacity (Nijhof and Bovendeur, 1990). However nitrite concentration was never found to build up in the nitrifying consortia during mass production, activation or during operation of the reactors indicating that two step nitrification under experimental conditions could be initiated. Though the nitrite oxidizers displayed reproductive rates lower than the ammonia oxidizers (Metcalf and Eddy, 1991; Paller, 1992) they were generally recognized as having higher oxidation rates than ammonia converters and thus low nitrite levels are typical in a balanced filter (Bovendeur, 1989).

4.3.3.4 Effect of organic carbon on nitrification

Particulate organic matter negatively affect nitrification in biofilters by way of clogging, occupying surface area to be colonized by bacterial biomass as well as the through the addition of organics into the bulk volume (Wheaton et al., 1994). When high amounts of easily degradable organic matter are present in a biofilter, the fast growing heterotrophic bacteria will out-grow the slow growing nitrifiers from the aerobic zone in the biofilm as they compete for oxygen and space (Wik and Breitholz, 1996; Wik, 2003). In the present short term experiments using the

SBSBR, the effect of sudden organic loading to the reactors was analyzed at C/N ratio of 0, 2 and 4. Average TAN, NO_2-N and NO_3-N concentrations after 1 hr in Stringed Bed Suspended Reactor deployed at C/N ratios of 0, 2 and 4 at salinities of 15 and 30 ppt are presented in (Fig. 11a -11c) and (Fig. 11d-11f) (n=9) respectively. The average percent TAN removal for each case was calculated (Fig.12). Even though there was a slight decrease in the percentage TAN removal with increasing order of C/N ratio, they were not statistically significant. This may be due to the short time period of the experiment insufficient for the heterotrophs to out compete nitrifiers in the reactors. Taking into consideration the downward trend observed in this short term experiments supported with the studies conducted in this area elsewhere a minimal organic loading in to the RAS for a steady state nitrification processes is essential. This can be achieved employing filters for the removal of particulate organic matter from the system as well the application of bioaugmentors.

4.3.3.5 Fluorescent in situ analysis of the mature biofilm

Prominent biofilm formation was observed on the beads taken from the reactor after completing an operating period of 6 months. (Fig. 13). FISH analysis of the biofilms with bacterial probe EUB338 and DAPI staining revealed the diversity of the bacterial cells forming the biofilms (Fig. 14 a and b). The probes for the β ammonia oxidizers (NSO 190) (Fig. 14c), *Nitrosococcus mobilis* lineage (NmV) (Fig. 14d), *Nitrobacter* sps (NIT2) (Fig.14e) and for the phylum *Nitrospira* (Ntspa 712) (Fig. 14f) have given positive signals from the biofilms. All the species identified in the biofilm were present in the consortia used for activating the reactors except *Nitrospira*. This proved the usefulness of the activated consortia to establish to a mature biofilm under real life situation. . The *Nitrospira* population observed in the biofilm might have developed from the recirculating water during the time course of operation. This also showed that the plastic beads used as carrier material was well suited for the establishment of nitrifying biofilms in practical sense. Structure and activity of multiple nitrifying bacterial populations in a biofilm was studied previously by several researchers using the

FISH probes and microelectrodes (Okabe et al., 1999; Schramm et al., 2000; Gieseke et al., 2003). Schramm et al. (2000) studied the distribution of nitrifying bacteria *Nitrosomonas, Nitrosospira, Nitrobacter* and *Nitrospira* in a membrane-bound biofilm system with supply of oxygen and ammonium from opposite directions, in which oxic part of the biofilm ,which was subjected to high ammonium and nitrite concentration was dominated by *Nitrosomonas europaea* like ammonia oxidizers and by members of the genus *Nitrobacter*, where as *Nitrosospira* and *Nitrospira* were abundant at the oxic-anoxic interface of the biofilm. In the totally anoxic part of the biofilm, cell numbers of all nitrifiers were found relatively low. In the present case the reactor system was operated with O_2 at saturation and a low TAN concentration of maximum of 2 mg/L. Less reports are available for the nitrifying bacterial populations inhabiting the biofilm having a limited supply of the substrates.

Yossi Tal et al. (2003) characterized a nitrifying microbial consortium from a moving bed bioreactor (MBB) connected to a marine recirculating aquaculture system using DGGE of amplified 16S rRNA gene fragments. The ammonia oxidizer *Nitrosomonas cryotolerans* and nitrite oxidizer *Nitrospira marina* were found associated with the system as well as a number of heterotrophic bacteria, including *Pseudomonas sp.* and *Sphingomonas sp.* and two *Planctomycetes sp.* were detected in the system suggesting the capability for nitrification, denitrification and annamox in the single system. Similar to this we have also observed a major non-nitrifying population in the biofilm, but the annamox probe gave no positive signals from the biofilm. This may be because of the highly aerated conditions in the system. We also observed a denitrification activity which kept the nitrate concentration always bellow 10 mg/L.

The information generated in this study on the biofilm bacterial communities of nitrifiers immobilized on the substrata can used to enhance the efficiency of the bioreactors by optimizing the operating conditions such as oxygen levels, water flow, temperature and nutrient load to induce a desirable process that is necessary

for optimal performance. The biofilms investigated showed the presence all constituent members present in the original consortia used for activating the reactors. This showed the stability of the consortia on long term operation. The kinetic studies showed that the substrate removal rates increased linearly with the substrate concentration. However, more studies at different spatiotemporal scales are required for a full scale evaluation of the SBSBRs and PBBRs developed. The biofilter/bioreactor performance studies are difficult to be conducted due to the large number of parameters that must be controlled and the number of measurements that must be completed. The validation of the bioreactors at field level in Recirculating Aquaculture Systems with feed and biomass loadings is the next step for evaluating the nitrification capacity of the bioreactors.

Table1. Filter and media characteristics of the packed bed bioreactor (PBBR)

Filter height	0.45 m
Water height	0.40 m
Water discharge height	0.35 m
Filter volume	40 L
Total surface area of media	4.71 m^2
Total media volume	0.023 m^3
Specific surface area	205 m^2/m^3

Table2. Filter and media characteristics of Stringed Bed Suspended Bioreactor (SBSBR)

Filter height	10 cm
Water height	10 cm
Filter volume	0.75 L
Total surface area of media	0.0674 m^2
Total media volume	0.00045 m^3
Specific surface area	150 m^2/m^3

Table 3. Average TAN removal rates and percent TAN removals in PBBR operated at steady state (TAN_{in} = 2 mg/L) at different flow rates

Flow rate Q (L/hr)	HRT (min)	TAN removal rates ($gTAN/m^2/day$)	TAN removal (%)
600	2	49.43	21.56
400	3	41.69	26.00
200	6	33.04	48.00

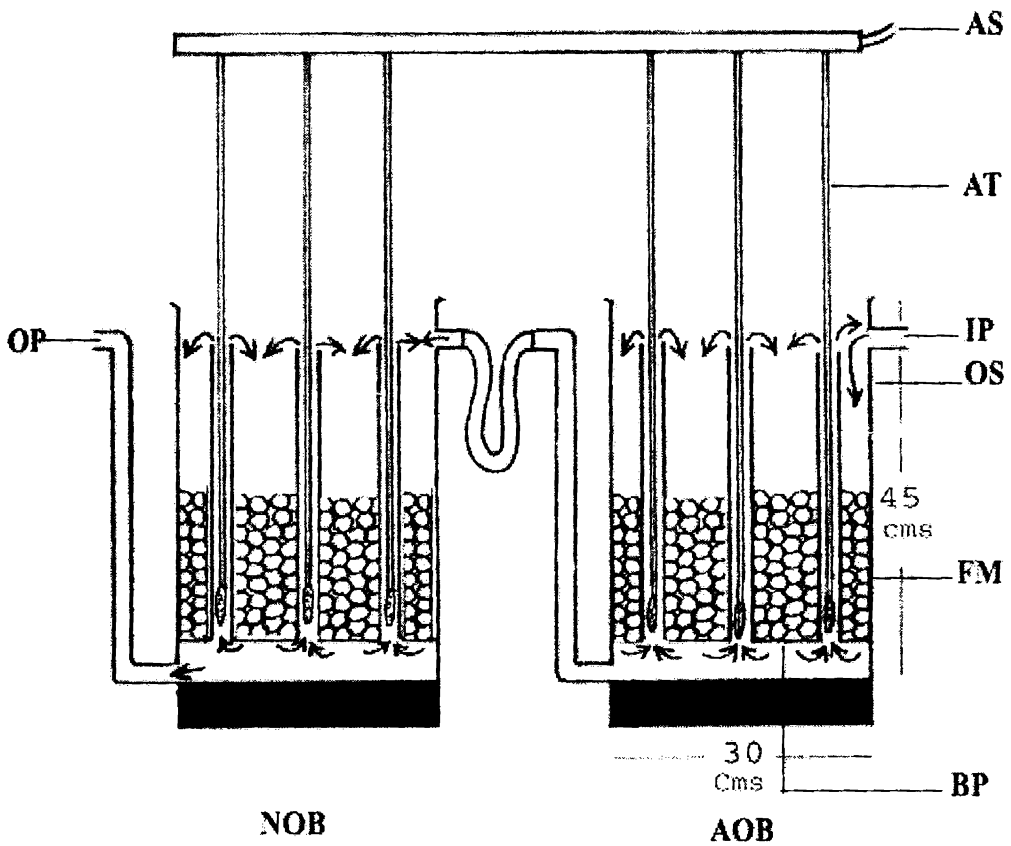


Fig.1. Cross sectional view of the bioreactors connected serially

(AOB-ammonia oxidizing bioreactor, NOB-nitrite oxidizing bioreactor, BP-base plate, FM- filter media, OS-outer shell, IP-inlet pipe, OP, outlet pipe, AT-aeration tubes, AS-air supply)

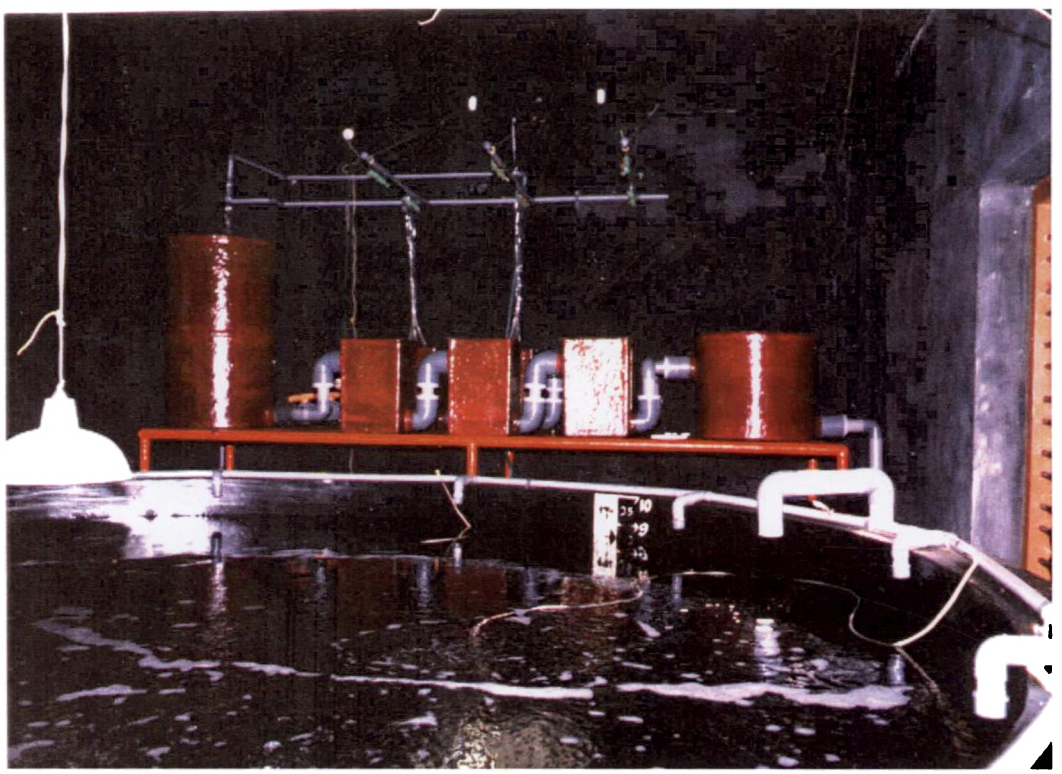


Fig.2. A fully assembled Packed Bed Bioreactor (PBBR) System

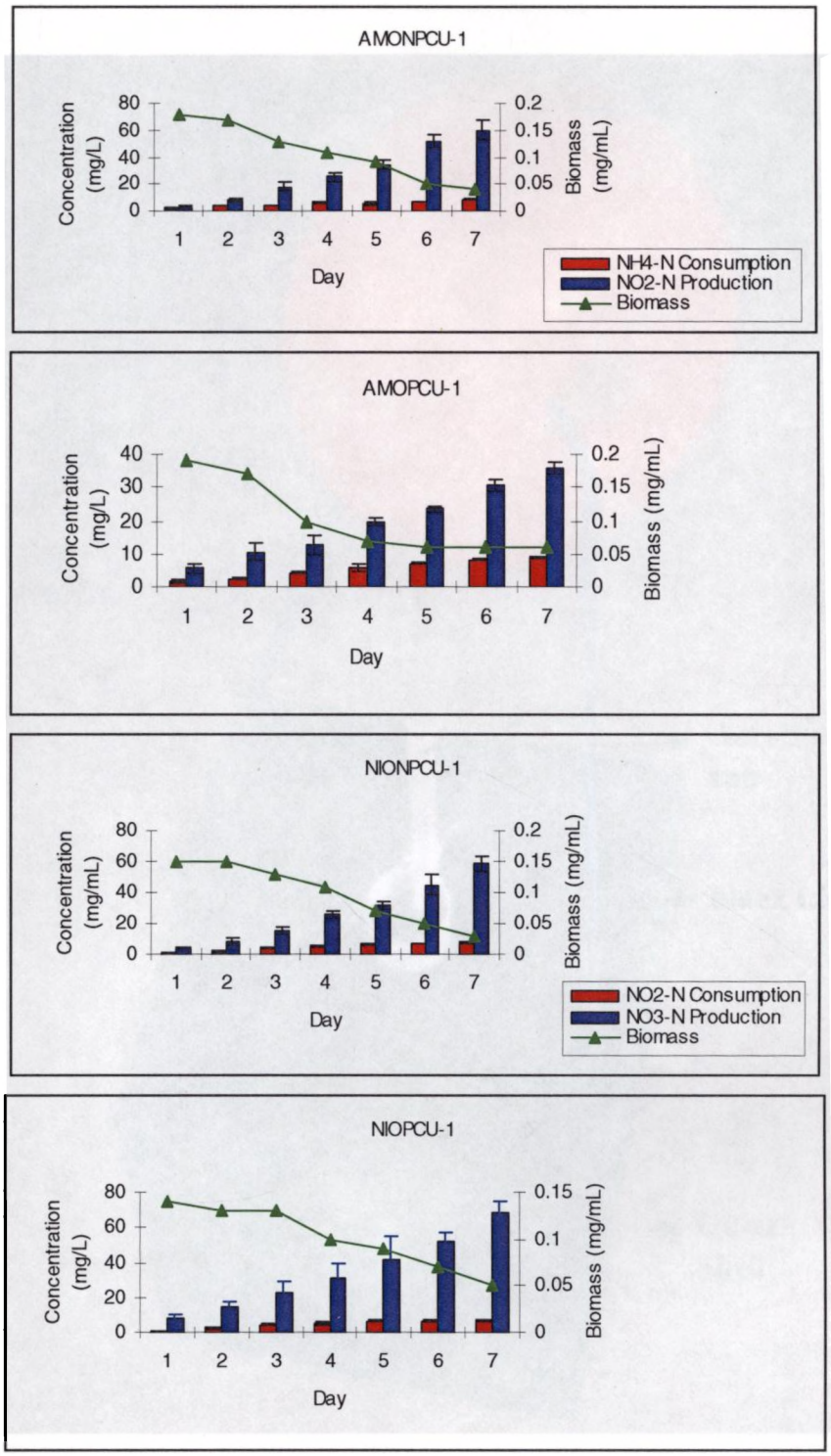


Fig. 3. Substrate consumption, product formation and biomass immobilization during activation of PBBR with ammonia (AMONPCU-1, AMOPCU-1) and nitrite (NIONPCU-1, NIOPCU-1) oxidizing consortia at 15 and 30 ppt respectively.

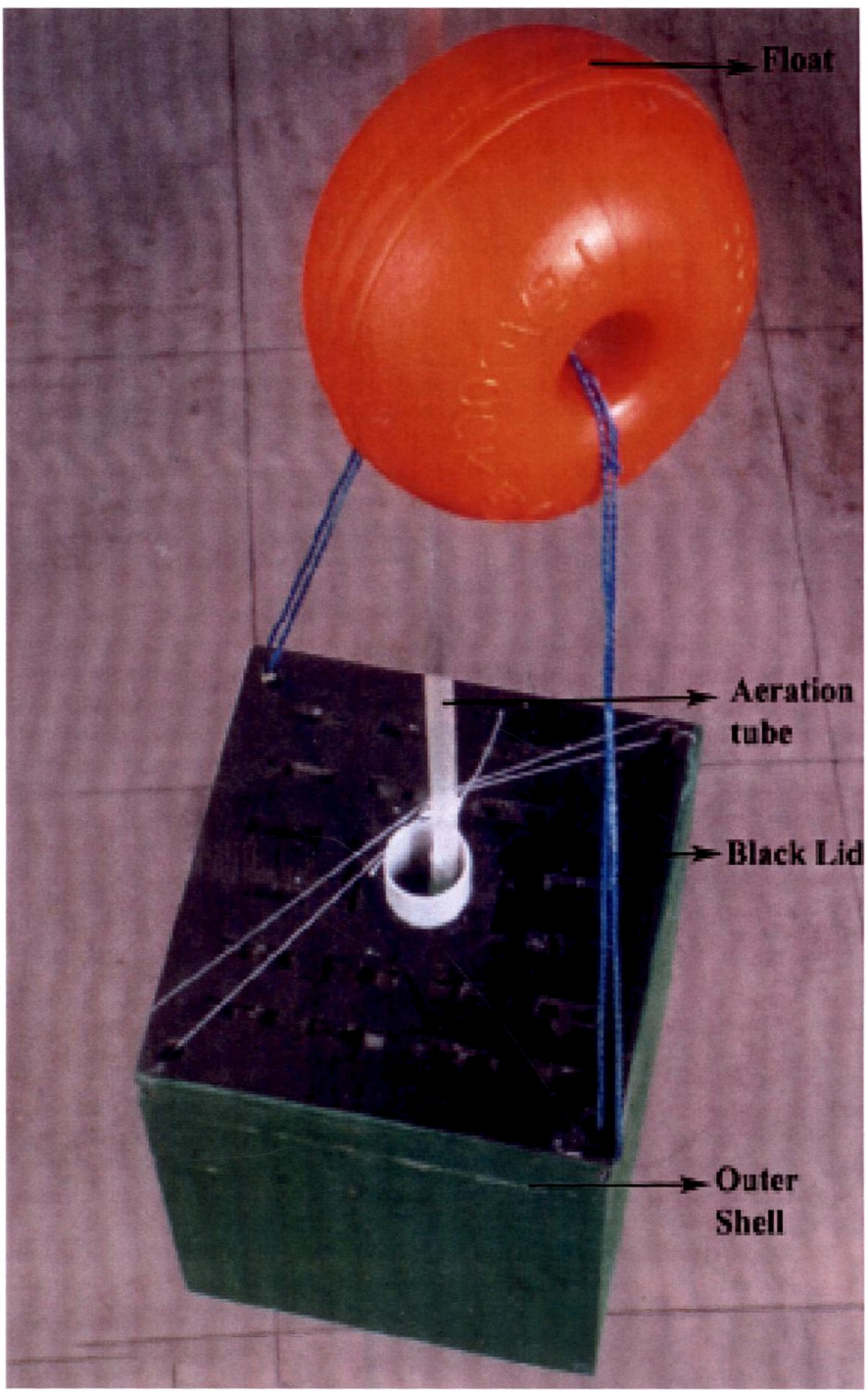


Fig.4. Stringed Bed Suspended Bioreactor on full assembly (SBSBR)

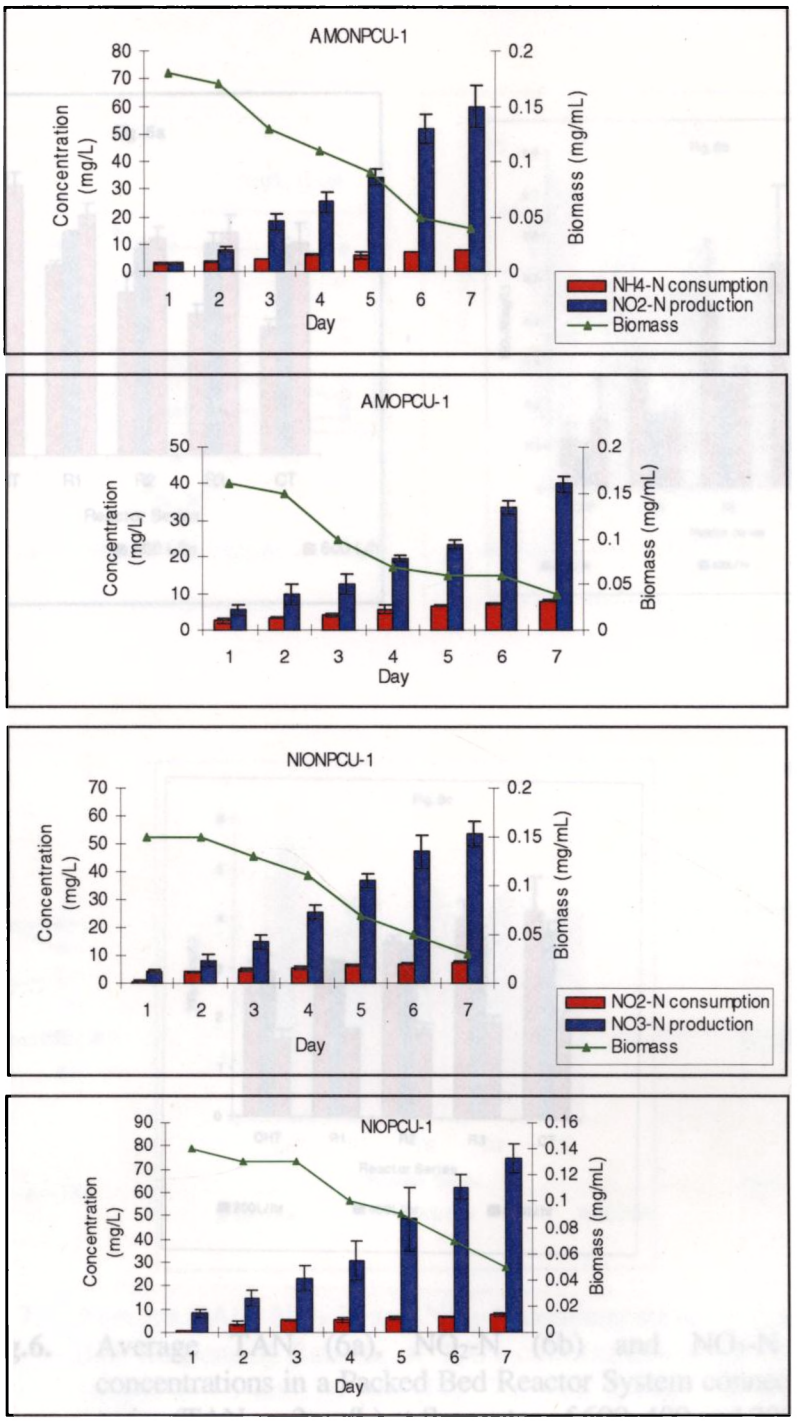
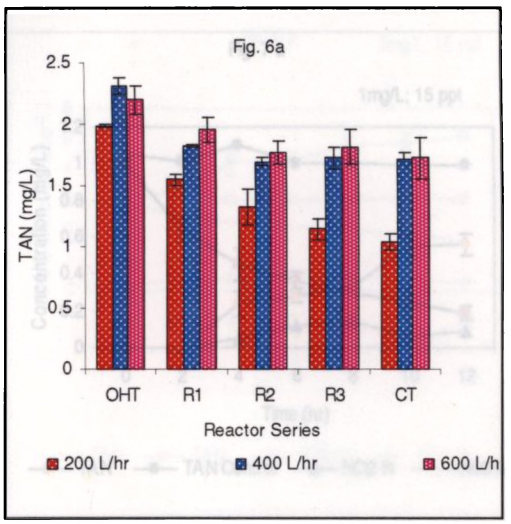
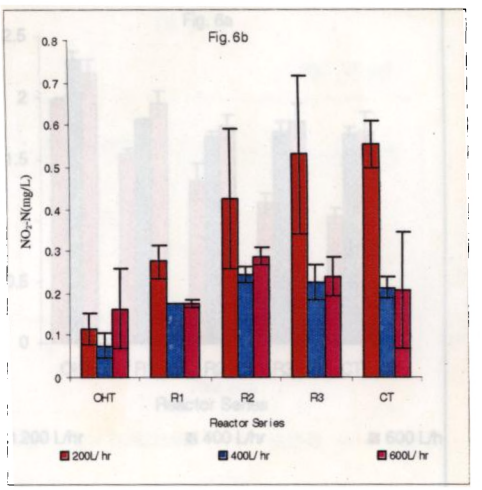


Fig.5. Substrate consumption, product formation and biomass immobilization during activation of SBSBR with ammonia (AMONPCU-1, AMOPCU-1) and nitrite (NIONPCU-1, NIOPCU-1) oxidizing consortia at 15 and 30 ppt respectively.





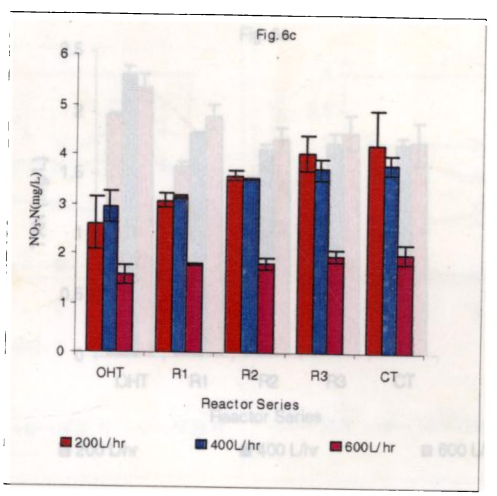


Fig.6. Average TAN (6a), NO_2-N (6b) and NO_3-N (6c) concentrations in a Packed Bed Reactor System connected in series (TAN_{in} = 2mg/L) at flow rates of 600, 400 and 200 L/hr

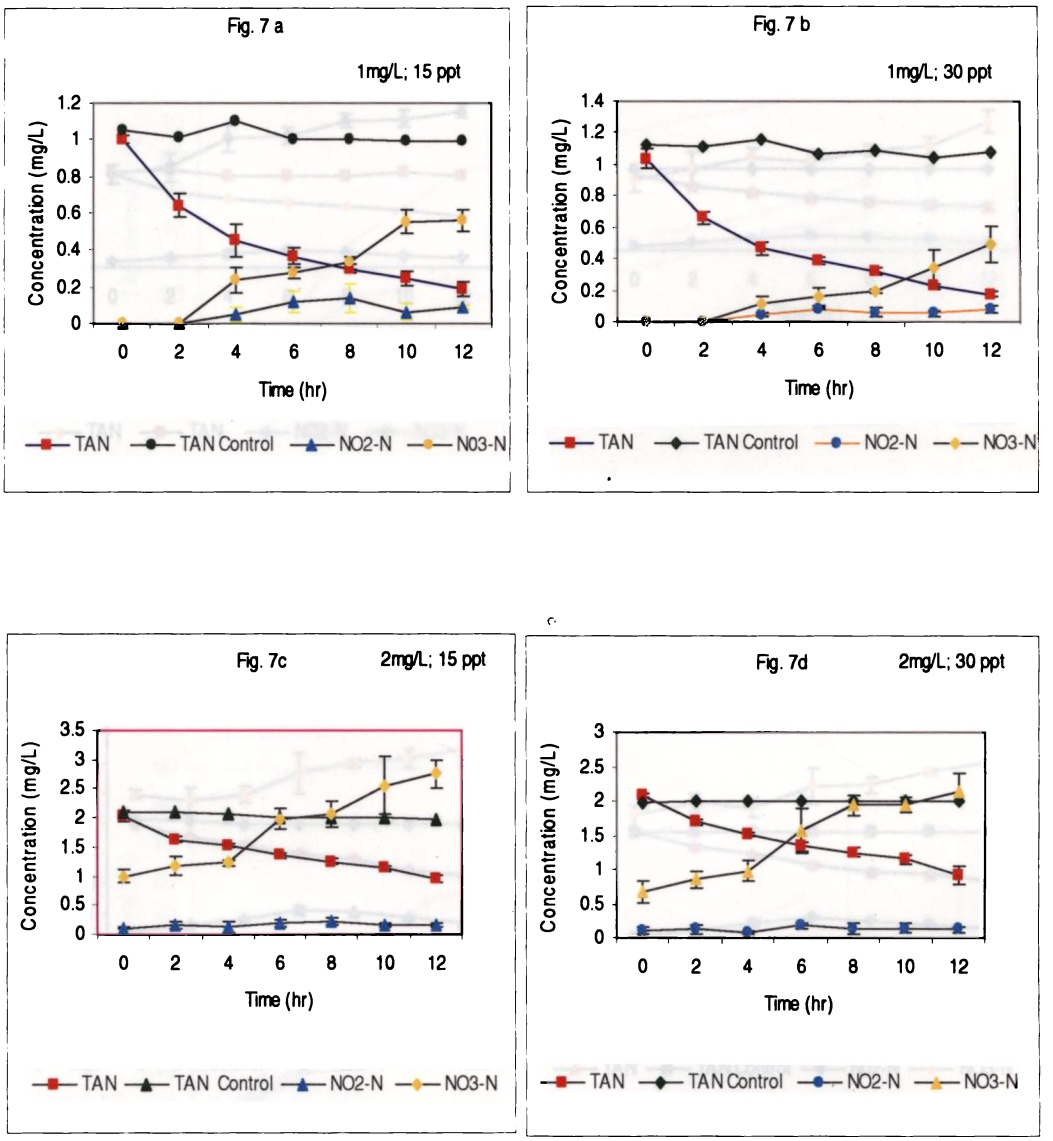


Fig. 7. Average TAN, NO_2-N and NO_3-N concentrations in Stringed Bed Suspended Reactor at TAN concentrations of (TAN_{in}) 1mg/L at 15 and 30 ppt (Fig. 7a, 7b), 2 mg/L at 15 and 30 ppt (Fig. 7c, 7d)

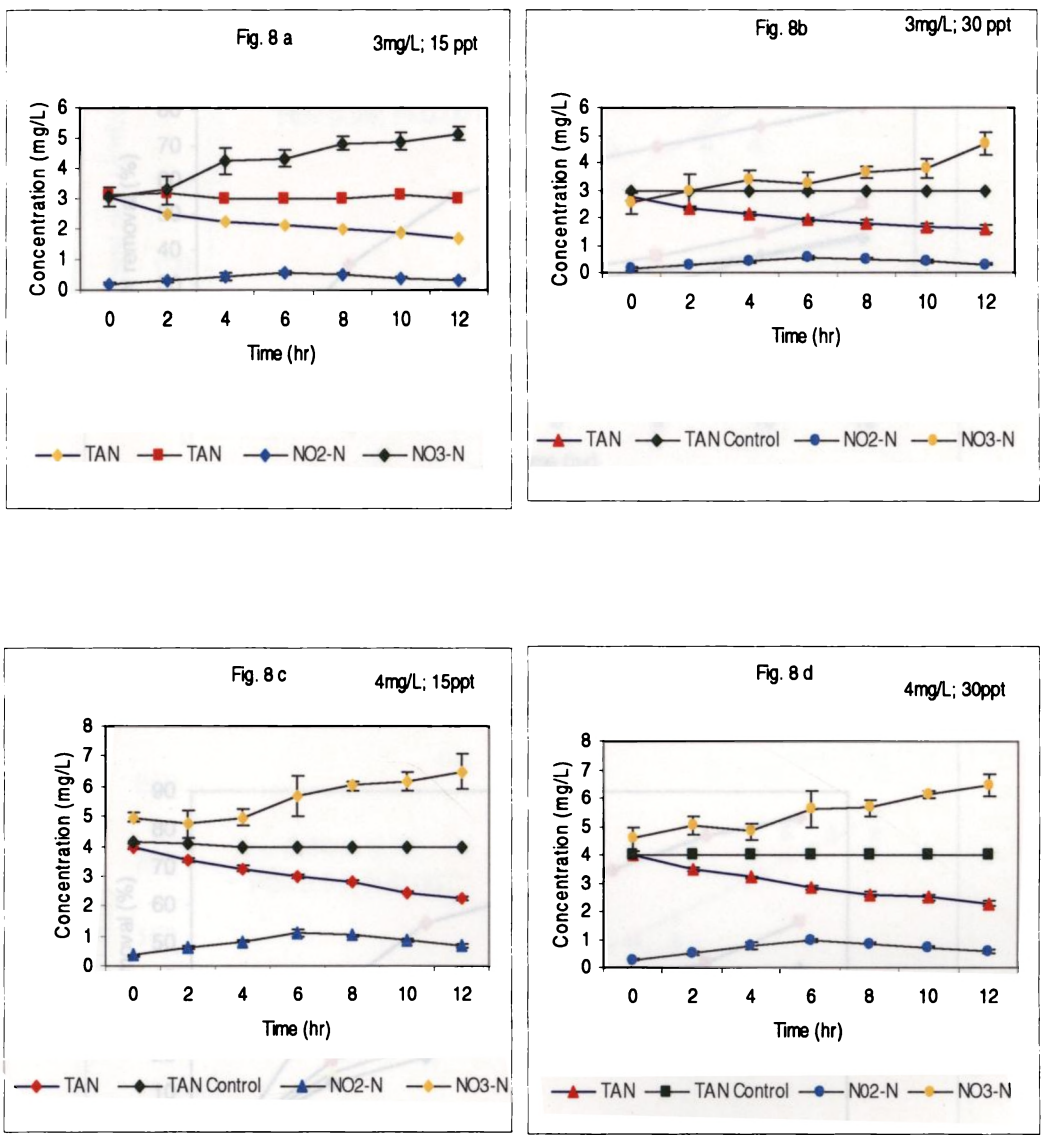
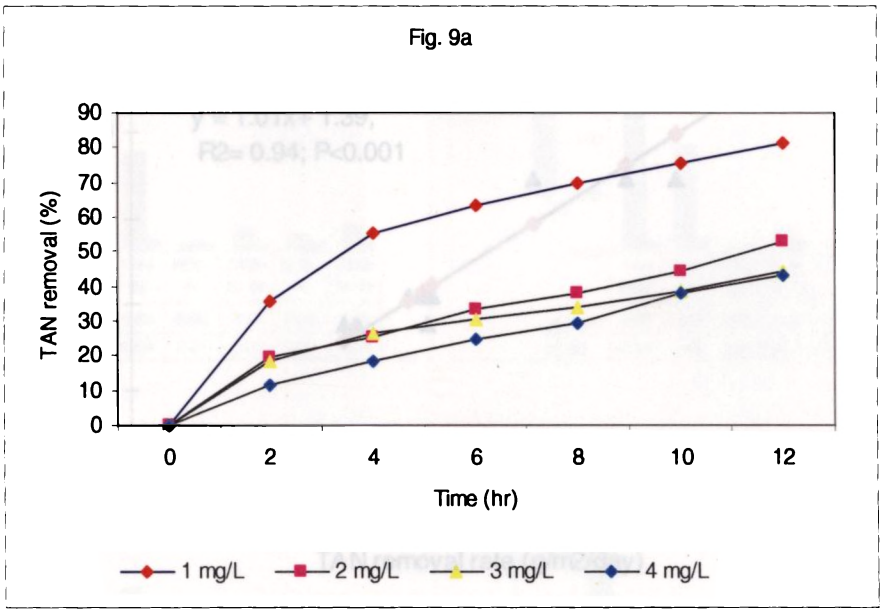


Fig. 8. Average TAN, NO_2-N and NO_3-N concentrations in Stringed Bed Suspended Reactor at TAN concentrations of (TAN_{in}) 3mg/L at 15 and 30 ppt (Fig. 8a, 8b), 4 mg/L at 15 and 30 ppt (Fig. 8c, 8d)



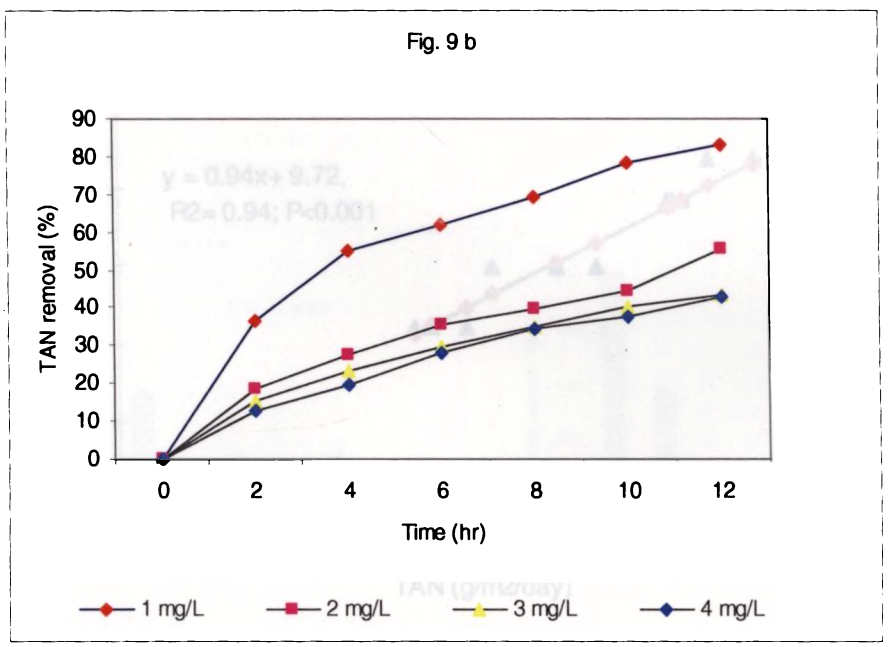
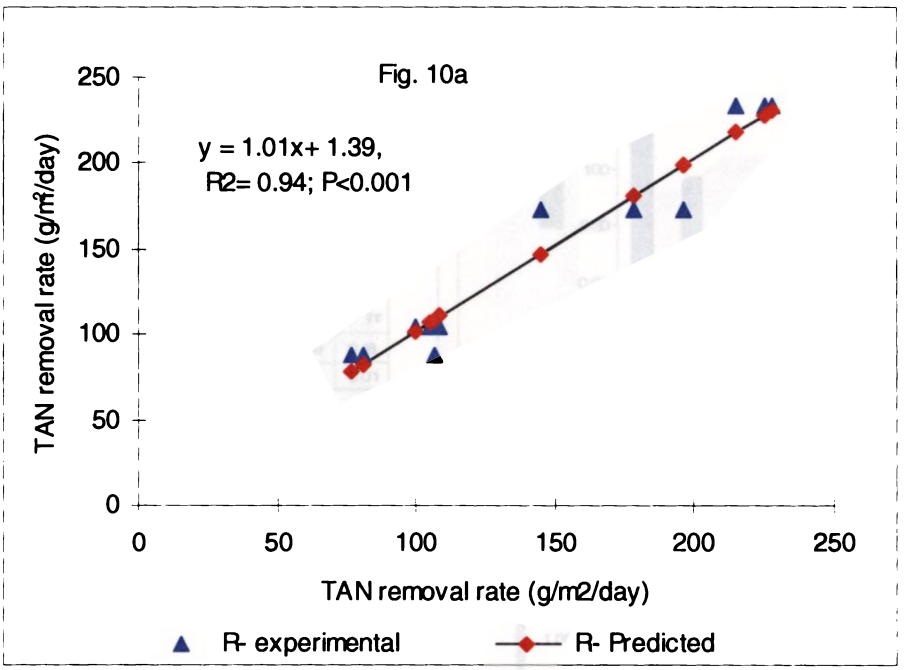


Fig. 9. Average TAN removal (%) in Stringed Bed Suspended Reactor at TAN concentrations of (TAN_{in}) 1, 2, 3 and 4 mg/L at salinities of 15 ppt (Fig. 9a.) and 30 ppt (Fig. 9 b)



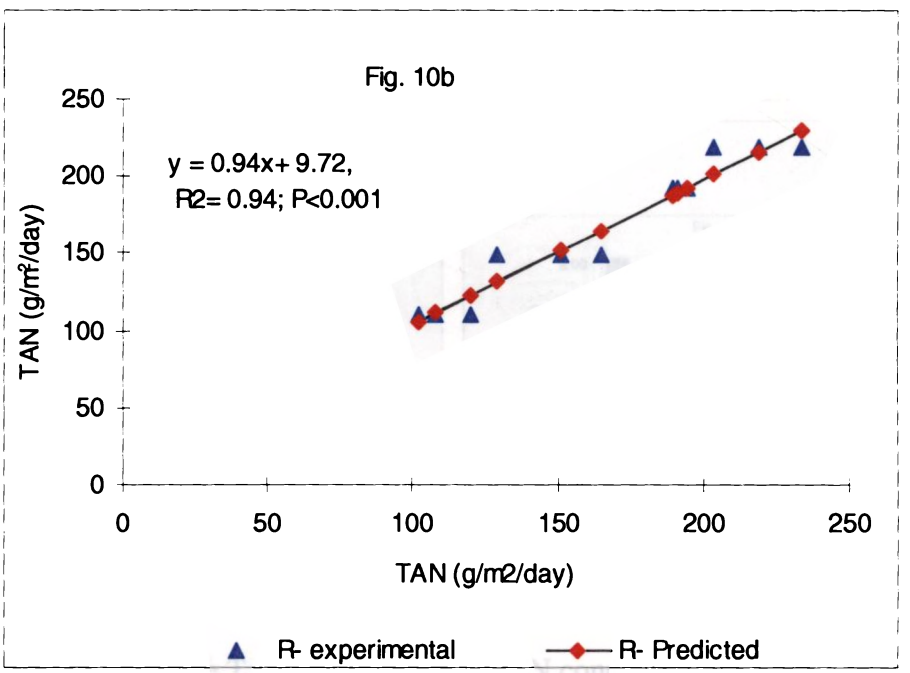
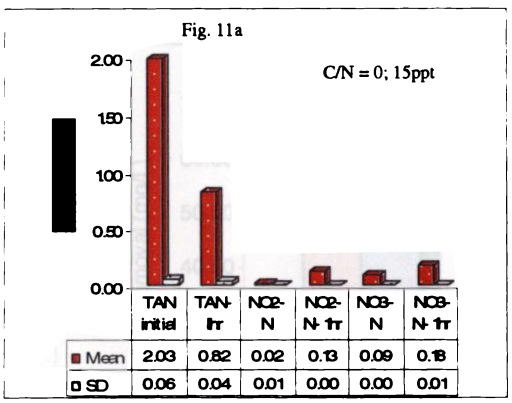
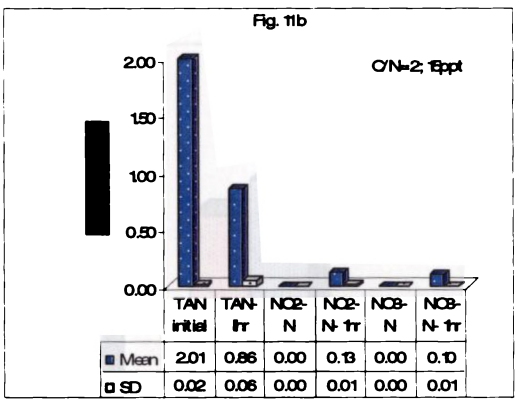
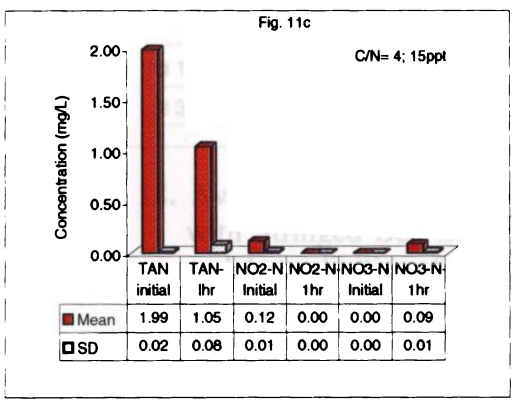
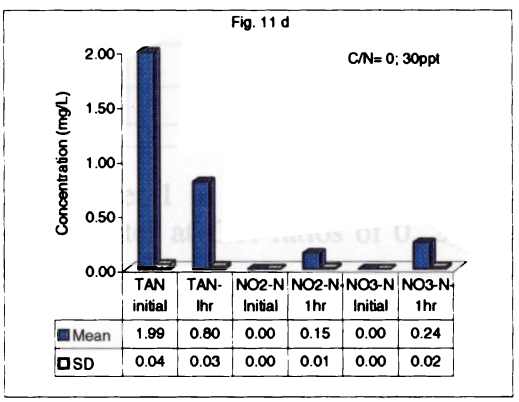


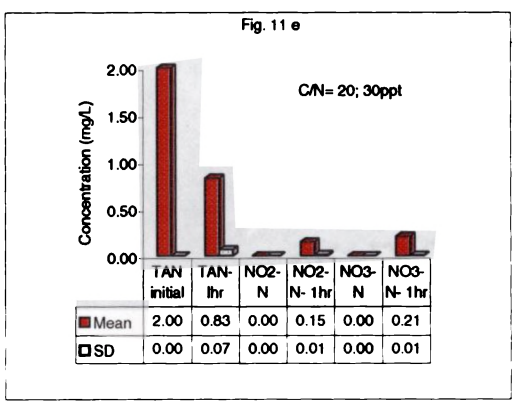
Fig 10. Average TAN removal rates in Stringed Bed Suspended Reactor at TAN concentrations of (TAN_{in}) 1, 2, 3 and 4 mg/L at salinities of 15 ppt (Fig. 10a.) and 30 ppt (Fig. 10 b)











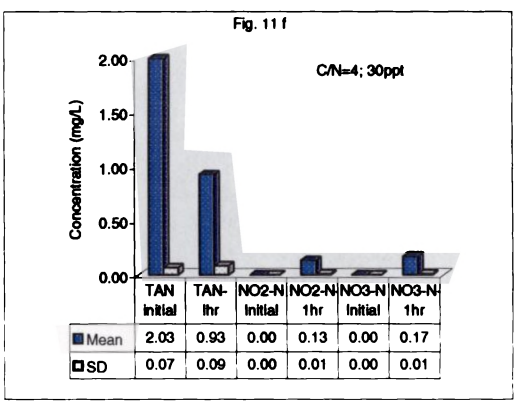


Fig. 11. Average TAN, NO_2-N and NO_3-N concentrations after 1 hr in media treated with Stringed Bed Suspended Reactor at C/N ratios of 0, 2 and 4 at salinities of 15 (Fig. 11a -11c) and 30 ppt (Fig. 11d-11f) (n=9)

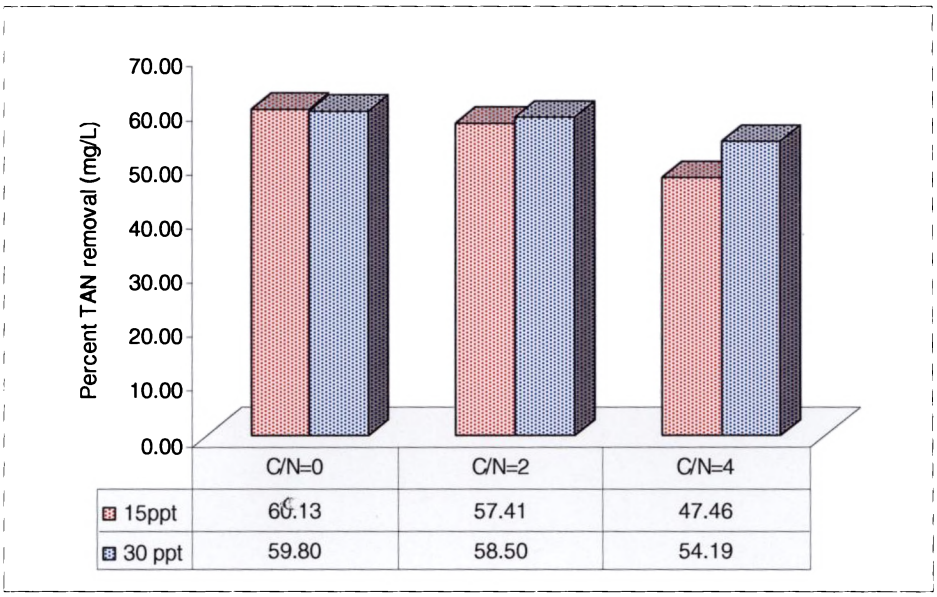
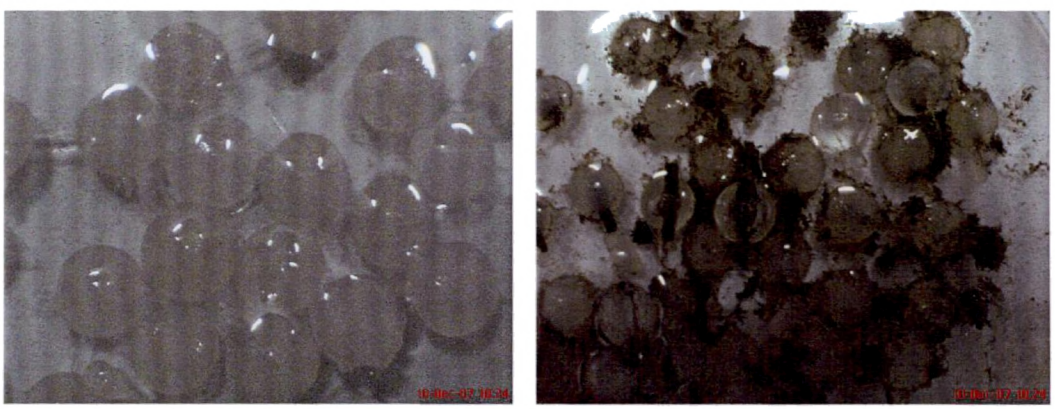
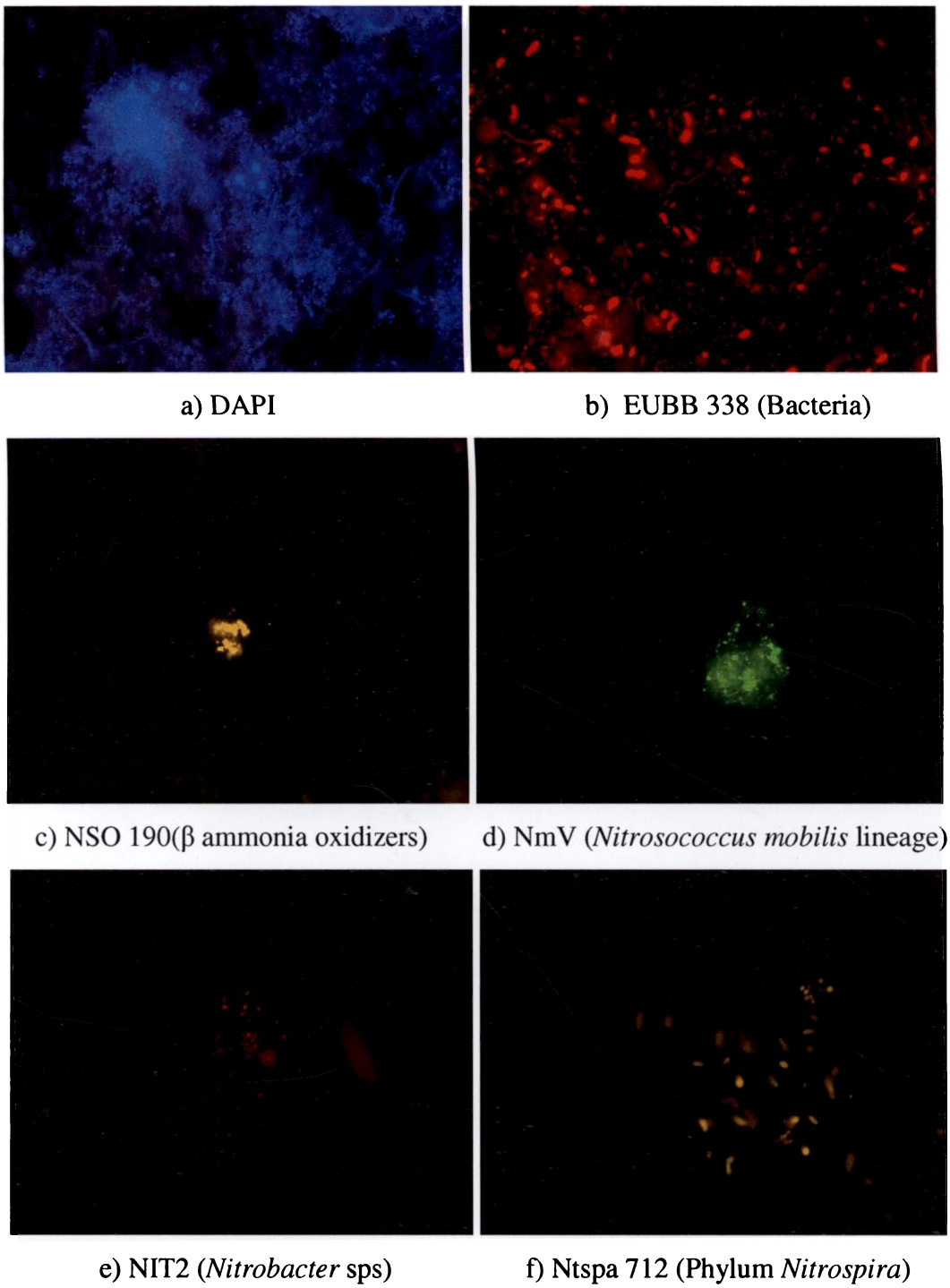


Fig. 12. Average Percent TAN removal after 1 hr in media treated with Stringed Bed Suspended Reactor at C/N ratios of 0, 2 and 4 at salinities of 15 and 30 ppt (n=9)



(a) (b)

Fig. 13. Polystyrene beads used as filter media
a) With out consortia
b) With a mature biofilm



a) DAPI b) EUBB 338 (Bacteria)

c) NSO 190(β ammonia oxidizers) d) NmV (*Nitrosococcus mobilis* lineage)

e) NIT2 (*Nitrobacter* sps) f) Ntspa 712 (Phylum *Nitrospira*)

Fig.14. Fluorescent Insitu Hybridization of a mature biofilm taken from PBBR originally activated with the nitrifying bacterial consortia AMONPCU-1 and NIONPCU-1, after 6 months of operation

Chapter 5

VALIDATION OF THE NITRIFYING BIOREACTORS IN RECIRCULATING AQUACULTURE SYSTEMS

Contents

5.1. Introduction
5. 2. Materials and Methods

- 5.2.1. Stringed Bed Suspended Bioreactors in recirculating aquaculture systems
- 5.2.2. Packed Bed Bioreactors in Recirculating Aquaculture Systems
- 5.2.3. A Recirculating Aquaculture System for *Penaeus monodon*: Laboratory Level Demonstration

5.3. Results and Discussions

- 5.3.1. Validation of Stringed Bed Suspended Bioreactors
- 5.3.2. Validation of Packed Bed Bioreactors
- 5.3.3. Laboratory Level Recirculating Aquaculture System for *Penaeus monodon*

5.1 Introduction

On assuming the dimensions of an industry, the aquaculture systems are bound to operate under strict environmental safety standards. Stringent regulatory guidelines focusing on discharges to natural water bodies will force hatchery operators to adopt methods that are environment friendly (White et al., 2004). With high land and water costs, these systems are designed to maintain high biological carrying capacity in relatively little space with minimal water exchange. Recirculating Aquaculture System (RAS) can reduce the effluent waste stream by a factor of 500-1000 (Chen et al., 1997; Timmons et al., 2001) allowing existing operations to upgrade and expand and comply with future regulations. RAS allow companies to 1) be competitive in both domestic and world commodity markets by locating production closer to markets, 2) improve environmental control, 3) reduce catastrophic losses due to diseases, 4) avoid violation of environmental regulations on effluent discharge 5) reduce management and labour costs, and 6) improve product quality and consistency (Lee, 1995). The use of RAS is gaining wide acceptance in view of the expanding marine production and the demand for biosecure systems to produce larvae, fry and fingerlings for grow out systems (Turk et al., 1997; Watanabe et al., 1998; Malone, 2002; Otoshi et al., 2003; Pruder, 2004). The water quality expectations for these systems can fall well below the 0.3 g-N/m^3 TAN standard set for the oligotrophic classification. Most marine hatchery systems require oligotrophic water quality conditions in order to maintain a healthy stock (Table.1). Larval systems typically require TAN concentrations less than 0.1 g-N/m^3 and fingerlings typically require a TAN concentration less than 0.5 g-N/m^3 (Malone and Beecher, 2000).

The high values associated with fingerlings and marine and freshwater ornamentals are also promoting adoption of recirculating technologies (Howerton, 2001; Palmtag and Holt, 2001; Gutierrez-Wing and Malone, 2006).

Table.1. Ideal water quality ranges for *Penaeus monodon* maturation, hatchery facility and hatchery effluent standards (FAO, 2007)

Parameters	Maturation/hatchery facility	Hatchery effluent standards
Temp(°C)	28-32	-
pH	7.8-8.2	6-9.5
Total ammonia-nitrogen(mg/L)	-	<5
Ammonia (NH_3) (mg/L)	<0.1	
Nitrite (NO_2) (mg/L)	<1	
Nitrate (NO_3) (mg/L)	<10	
Oxygen (mg/L)	>4	>4
Iron(mg/L)	<1	
Hydrogen Sulphide (H_2S) (mg/L)	<0.003	-
5-day BOD(mg/L)	-	<50

However, RAS for producing freshwater commodity food fish are unable to compete directly in terms of cost with pond or flow-through systems and this turns out to be the main obstacle for a wider adoption of RAS technologies in freshwater applications (Timmons and Losordo, 1994; Lorsordo and Westeman, 1994; Malone, 2002). While it may be relatively straightforward to culture large numbers of seed animals in hatcheries, the poor quality of juveniles may limit the effectiveness of any release programme (Vay et al., 2007) as the environmental conditions and husbandry practices within the hatchery as well as broodstock and larval nutrition happen to be the factors which influence the quality of offspring. Under such circumstances maintenance of environmental quality during larval rearing becomes critically important and RAS can satisfy such requirements provided the system is equipped with the necessary devices

Coming to shrimp culture, its future success as an industry is dependent on the increasing supplies of healthy, high quality seed for stocking ponds. All types of commercial penaeid larval culture systems have been plagued by problems with pathogenic microorganisms (Browdy, 1998). In these systems, high water exchange rates flush out bacterial populations destabilizing microbial community structure and opening up niches for more pathogens. With the greater demand for post larvae, there happened a cascade of events which lead to increased hatchery development, and higher stocking density opening up multitudes of problems in maintaining balance equilibrium of bacterial communities. Sterilization of incoming water in fact destroys all beneficial bacteria and opens niches for whichever relatively pathogenic or benign strain colonizes first. In most cases, antibiotics were used as the treatment of choice with well known negative consequences. These include elimination of both beneficial and pathogenic strains further destabilizing microbial communities and the development and spread of resistant strains which increase problems within hatcheries and pose potential public health concerns. A study by Otoshi et al. (2003) indicated that broodstock shrimp can be cultured in a biosecure RAS while maintaining good growth and high survival. In addition, rearing broodstock in a biosecure RAS does not negatively affect reproductive performance and may facilitate the development of Specific Pathogen Free (SPF) captive breeding programmes. Many recirculating system designs for production of marine shrimp have been published (Mock et al., 1977; Neal and Mock, 1979; Reid and Arnold, 1992; Davis and Arnold, 1998; Tseng et al., 1998); however, are with limitations especially regarding total nitrogen removal. Menasveta et al. (1989, 1991), Chen et al. (1989) and Millamena et al. (1991) reported the development of closed, recirculating seawater systems for black tiger shrimp maturation.

M. rosenbergii, though an inhabitant of freshwater, its larval stages require brackishwater for growth and survival. Extensive research carried out in the seed production and culture led to the 'green water' method of seed production and many advances were also made in hatchery technology (Chowdhury et al.,

1993). Recirculating systems indeed have shown to give consistent production of quality post-larvae. In India *M. rosen*bergii is cultured in an area of 34,630 ha with an average production ranging from 880 to 1250 kg/ha. To support the scampi industry in India, there are 71 hatcheries spread across the country supplying 183 billion scampi seed (Bojan, 2007). Two decades ago, most hatcheries were operating on flow-through systems. However, with the establishment of inland hatcheries, the costs of obtaining and transporting seawater or brine, and increasing concerns about the discharge of saline water in inland areas have encouraged many to minimize water consumption through either partial or full recirculating systems (New, 2002). Consequently several attempts have been made to develop and optimize recirculation in aquaculture systems focusing on total ammonia nitrogen (TAN) as the key limiting water quality parameter (Losordo and Westers, 1994; Lysscnko and Wheaton, 2006).

An efficient biofilter is the central component in RAS to maintain the water quality. Proper selection and sizing of biofilters are critical to both the technical and economic success of a recirculating aquaculture system. To satisfy this requirement wide variety of biofilters are available and as such the variability of conditions under which a given biofiltration platform is expected to perform is a matter of concern (Malone and Pfeiffer, 2006). However, there will be an increase in demand for cost effective biofilters with expanded use of RAS (Gutierrez-Wing and Malone,2006), especially with oligotrophic and ultraoligotrophic technologies for marine nurseries and emphasis needs to be placed on their sizing in support of nursery operations. Biological filters are essential components of recirculation systems for freshwater prawn hatcheries also and many types of biofilters are available for use in freshwater systems (Valenti and Daniels, 2000; New, 2002). Some of the major limiting parameters in the development of biosecure, closed recirculating aquaculture filtration technology are the cost-effective removal of particulates and nitrogen from the recirculating water (Malone and DeLosReyes, 1997). As pointed out earlier, biofilter performance studies are difficult to be conducted due to the large number of parameters that must be controlled and the

number of measurements that must be completed (Colt et al., 2006). Nitrification capacity of a biofilter is complicated by the sensitivity of the nitrifying population to a variety of water quality factors (Belser, 1979). The loading history and environmental conditions to which the biofilm has been subjected needs to be given careful consideration when evaluating a filter's nitrification performance (Malone and Pfeiffer, 2006). The simplest rate of nitrification that can be calculated for a biofilter is simply the mass conversion rate of nitrogen from one form to another or the gross nitrification rate. The generation of ammonia within a recirculating system depends on the feeding rate, protein content of the feed, fraction of protein nitrogen that is excreted as TAN and the rate of TAN excretion.

Although great efforts have been made on the investigation of nitrifying biofilters for aquaculture applications, the research have mostly been focusing on performance of an individual component under specific operating conditions using average ammonia removal rate to describe the biofilter nitrification performance. The reported nitrification rates of biofilters varied among systems depending on operating conditions and ammonia loadings. The Volumetric TAN Removal Rates (VTR) is usually used to express the efficiency of the biofilter. The VTR does not require an estimate of the total active surface area of media and can be used directly for system design. Three statistical problems have been identified when reporting VTR values such as the statistical definition of steady-state TAN removal, the randomization of treatments, and the proper replication of experimental units during experimental design (Colt et al., 2006).

Previous studies have shown that an acclimation period is necessary before a biofilter begins to operate properly in marine systems provided they are not inoculated with pure or mixed cultures of nitrifiers. Normal acclimation times can be expected to be of the order of 2–3 weeks for freshwater systems (Masser et al., 1999). However marine systems frequently seem to stop the nitrification in the ammonia oxidizing stage of acclimation. It is not uncommon to see systems with persistent nitrite accumulations for periods as long as 3–4 months (Manthe and

Malone, 1987). It may take 60 days for a new filter to approach steady-state conditions, especially with some category of plastic media in sea water (Colt et al., 2006). However, the Packed Bed and Stringed Bed Suspended reactors described in the previous chapters were found to set up instant nitrification on integrating the activated reactors into tropical hatcheries making theses systems 100% closed recirculation systems (Singh et al., 2007).

The recirculating aquaculture systems are usually operated as closed systems and many essential nutrients progressively getting depleted from water column are replaced/replenished from the digestive/excretory metabolites or feed contaminants and subsequently get accumulated to toxic levels with time (McNeil, 2000). This suggests the requirement of waste management in recirculation systems, a serious concern, as it leads to problems connected with bad water quality and disease outbreak. To tide over the situation the practice of applying viable bacteria and their products has been introduced to regulate water presuming that the added bacteria produce greater quantities of a range of exoenzymes breaking down organic compounds (Moriarty, 1997). Moreover the added organisms stabilize or enhance a microbial community in the gastrointestinal tract and within the culture system favourable to the animal, so as to improve growth, survival, and disease resistance (Douillet, 2000; Karunasagar et al., 2000; Sonnenholzner and Boyd, 2000; Horowitz and Horowitz, 2001). Adequate scientific evidence could be cited concerning the beneficial effects of probiotics in clear water hatchery conditions (Fegan, 2000). Our previous studies have shown that 'Detrodigest', an indigenous probiotic preparation containing the bacterium *Bacillus* MCCB101 (GenBank accession no. EF062509), 'Enterotrophotic' a gut probiotic preparation composed of *Bacillus* MCCB 101 and *Micrococcus* MCCB 104 (Jayaprakash et al., 2005) could maintain the water quality and animal health and control the *Vibrio* population in penaeid and non-penaeid culture systems. Therefore, the operation of biofilters along with the application of indigenous probiotics can pave the way for a well defined biosecure recirculation larval production system that totally eliminates application of chemical disinfectants,

antibiotics and other chemotherapeutic agents facilitating organic shrimp/prawn seed production (Singh et al., 2004). In the present study both SBSBR and PBBR were validated under various conditions. Validation of SBSBR was accomplished under laboratory conditions in the maintenance of adult and brood stock of *Penaeus monodon* and larval production systems of *P.monodon* and *Macrobrachium rosenbergii*. Meanwhile PBBR was validated in a *P.monodon* commercial maturation system, *M.rosenbergii* larval production system and laboratory level recirculating system for intensive post larval rearing to juveniles to obtain disease free animal for study.

5. 2 Materials and methods

5.2.1 Stringed bed suspended bioreactors in recirculating aquaculture systems

5.2.1.1 Laboratory maintenance of *penaeus monodon* adults and brooders

Under this series of study two categories of experiments were conducted. In the initial case adults of *P.monodon* which were brought to the laboratory for various experiments were maintained without water exchange after deploying SBBRs. The animals (10 numbers) weighing 20 g each were maintained in a volume of 150 L at a salinity of 15 ppt supported with 2 SBSBRs. The animals were fed with pelleted feed together with steamed clam meat. The bioaugmentor Detrodigest and antagonistic probiotic *Micrococcus* MCCB 104 were applied at the rate of 10 mL per week. 'Detrodigest' is an indigenous probiotic preparation containing the bacterium *Bacillus* MCCB101 (GenBank accession no. EF062509) having 10^9-10^{12} cells/mL. Alkalinity destruction due to nitrification was compensated by the addition of Ca_2CO_3 as and when required. The shrimps were maintained for more than a month till they were taken for experiments.

In the second category of experiments *P. monodon* brooders (4) weighing approximately 150 g were maintained in a 200L tank integrated with 2 SBSBRs at 32 ppt salinity for 30 days. The brooders were fed intermittently with polychaetes (15g), with pelted feeds (5 g) and steamed clam meat (10 g) in total a day. As described above Detrodigest and *Micrococcus* MCCB 104 were applied at the rate of 10 mL per week. Here also alkalinity destruction due to nitrification

was compensated by the addition of $CaCO_3$ periodically. The brooders were maintained for a period of one month.

In both the above systems TAN, nitrite and nitrate concentration pH, temperature alkalinity and hardness were monitored every day. The heterotrophic bacterial community of the rearing water was determined once in a week by standard spread plate method employing ZoBell's Marine agar 2216 E prepared in seawater of salinity 30ppt. *Vibrio* counts were estimated using TCBS Agar plates.

5.2.1.2 Larval production of *Macrobrachium* rosenbergii and *Penaeus monodon*

The reactors were validated in two larval production systems such as *P. monodon* hatchery system of Matsyafed, Ponnani, Kerala and *M. rosenbergii* Hatchery of M/s Rosen Fisheries, Trichur, Kerala. As the systems were operating under two saline conditions such as 30 and 15 ppt the reactors were activated with AMOPCU-1 and NIONPCU-1 and; AMONPCU-1 and NIONPCU-1 respectively. Prior to transportation of the activated reactors, the aerosol arrestors were removed, the black lid on top of the reactor replaced and the medium drained off leaving about 250 mL to maintain moisture inside and tied securely in a polythene bag. On reaching the site the reactors were suspended from a float through a string 1 foot below the water level. Two tanks of 2000 L capacity each were maintained, one with reactors and the other as control without the reactors. After deploying the reactors to the larval rearing tanks they were connected to the air supply of hatchery and flow regulated using an airflow meter, to 1 L/ min. In this mode, water enters the reactor through the perforations on top of the black lid, passes through the cartridge, and comes out through the airlift pump.

In the *M. rosenbergii* larval production system, the two 2 tonne tanks brought under the study were initially with 250L filtered diluted seawater having a salinity of 15 ppt, stocked with 1,00,000.0 mysis each. The tank without the reactors served as control .The reactors (2 ammonia-oxidizing and 2 nitrite-oxidizing) were deployed and the physical, chemical and biological parameters were

quantified once in three days for 17 days. Every day addition of 200L filtered, chlorinated-dechlorinated seawater having salinity 15 ppt for 10 days brought the water level to the maximum capacity of 2 tonne. During this period the larvae were fed with freshly hatched *Artemia* nauplii. It took 30 days for larvae to metamorphose to post larvae.

In *P. monodon* hatchery the same operation was adopted except in having seawater with 30 ppt salinity instead of 15ppt. Larvae were fed with *Chaetoceros*, *Spirulina* and commercial Zoea/Mysis feed and at post larvae with newly hatched *Artemia* nauplii.

During the period of larval rearing in both the systems no water exchange was provided. The experiment was repeated three times. Physical and chemical parameters measured during the experiment were salinity (using Refractometer, Erma-Japan), pH (using pH probe, Euteck-Singapore), ammonia (Solorzano 1969), nitrite (Bendschneider and Robinson 1952), nitrate (Strickland and Parsons 1972), alkalinity and hardness (APHA, 1998). Heterotrophic bacterial population was estimated following standard spread plate method employing ZoBell's agar 2216 E prepared in seawater of corresponding salinity. The reactors after experimentation were brought to the laboratory and tested for their activity by measuring the substrate uptake of the reactor over a period of 24 hr. On completion of the experiment (when the post larvae of *M. rosenbergii* attained the stage PL 5 and *P. monodon* at the stage PL 15), overall survivals in both the control and experimental sets of tanks were estimated numerically. Relative per cent survival was calculated according to the equation (Gram et al., 1999):

$$RPS = \left(\frac{1 - \%\ \text{Mortality in the tank}}{\%\ \text{Mortality in the control}} \times 100 \right) \quad \text{.....(5.1)}$$

The validation experiments were performed three times and the data analyzed statistically. One way Analysis of Variance was performed to test the significance of nitrification and survival rates in the control and test tanks

5.2.2 Packed bed bioreactors in recirculating aquaculture systems

5.2.2.1 *Macrobrachium rosenbergii* seed production system

The reactors were activated with the consortia AMONPCU-1 and (NIONPCU-1) (Achuthan et al., 2006) and integrated into a *Macrobrachium rosenbergii* Hatchery of M/s Rosen Fisheries, Trichur, Kerala. The facility used consisted of two larval rearing tanks of 5000 L capacity, one integrated with the activated reactors and the other without any, used as control. The ammonia oxidizing and nitrite oxidizing reactors were connected serially (Fig. 1). The influent from the rearing water tank was pumped in to an overhead tank (282 L) from where water flowed through the two reactors serially by gravitation and got collected in a 140 L collection tank. From the outlet of the collection tank treated water got in to the larval rearing tank. Pumping of the influent from the larval rearing tank was controlled by an automated water level controller (V-guard, Kerala, India) fitted inside the overhead tank. A regulator valve was connected to the overhead tank to maintain a flow rate of 4 L/min to the system attaining a total circulation of 5760 L/day.

Chlorinated-dechlorinated seawater of salinity 15ppt was used during the trial period. The tanks were maintained with 2000 L seawater. Fresh hatched mysis of *Macrobrachium rosenbergii* before their introduction to the culture system were disinfected by dipping in 0.025 mg/L formalin (SRL, Mumbai, India) for 20 seconds, 0.03 mg/L iodophore (Growel Formulations, Hyderabad, India) for 20 seconds and then washed in running seawater and stocked at 0. 2 million/ tank (200/L).

During the experiment, the rearing water was supplemented with 1 mg/L EDTA (Matrix Formulations, Hyderabad, India), 5 mg/L sulphated vitamin C (Matrix Formulations, Hyderabad, India) and 1 mg/L treflan (Growel Formulations, Hyderabad, India). The larvae were fed with fresh hatched *Artemia* nauplii up to stage 9 (when pleopods with setae appear) and with both *Artemia* nauplii and egg custard subsequently. The experiment continued for 17 days till the larvae metamorphosed to post larvae, and repeated two more times for concurrent

results. At the end of the experiment the survival was estimated by counting the larvae manually and the relative percentage survival was estimated as described earlier.

In another experiment, the reactor was tested for its nitrification potential in spent water after the larval culture. Water from the larval rearing tanks, subsequent to harvest of post larvae, was collected and stored in a 5000 L capacity storage tank. This was subsequently circulated through the bioreactor assembly at a rate of 2 L/min. Meanwhile, another system without integration of the reactor was kept as the control. The experiment was repeated two times.

Water samples from the larval rearing tanks were analyzed once in three days for alkalinity, hardness, ammonia (TAN), nitrite and nitrate. The heterotrophic bacterial community of the rearing water was determined once in a week by standard spread plate method employing ZoBell's Marine agar 2216 E prepared in seawater of salinity 15ppt.

In spent water nitrification experiments, water quality parameters such as phosphate, sulphate, iron, chloride, dissolved oxygen, Biochemical Oxygen Demand (BOD) (APHA,1998) and ammonia, nitrite and nitrate as above were estimated for eight days.

The nitrification efficiency of the control and reactor integrated hatchery systems was analyzed by one way ANOVA. Significance of percentage survival of larvae in the control and reactor integrated tanks was estimated by one way ANOVA, and Least Significant Difference (LSD) at 0.1% level calculated for the delineation of the two treatments. The mean and standard deviation of the water quality parameters of the spent water were estimated.

5.2.2.2 *Penaeus monodon* maturation system

The activated Packed Bed Bioreactors were integrated into a maturation system operated at West Coast Hatchery, Alapuzha, Kerala, India. The maturation tanks were filled with 5 tone 30ppt seawater. The bioreactor was operated at a flow rate

of 400L/ min. The tanks were stocked with 40 eyestalk ablated brooders with an average weight of 150 g. The animals were fed with clam, squid and crabs 3 times a day. The bioaugmentor Deterodigest and the gut probiotic Enterotrophotic were applied in to the system at a quantity of 100mL once in 3 days. 'Enterotrophotic' is a gut probiotic preparation composed of *Bacillus* MCCB 101 (GenBank accession no. EF062509) and *Micrococcus* MCCB 104 (Jayaprakash et al., 2005), blended in equal proportion to attain 10^8-10^9 CFU/ mL. The water samples were analyzed daily for pH, alkalinity, total ammonia, nitrite and nitrate following the methods described earlier. Vibrio was enumerated once in three days by plating on TCBS agar.

5.2.3 A Recirculating aquaculture system for *Penaeus monodon*: laboratory level demonstration

A laboratory level recirculating culture system for *Penaeus monodon* was established at this Centre. A series of six activated packed bed bioreactors 3 each in parallel were integrated in to system where in 1000L rearing water was recirculated through the reactors making its 100% recirculating. The reactors were activated with the consortia AMONPCU-1 and NIONPCU-1. The tanks were filled with sea water of salinity of 15 ppt. The reactors were operated at a flow rate of 4L/min. A bag filter was used to filter the incoming water from the rearing tanks to remove detritus. The larvae (PL-20) were tested for WSSV by PCR and MBV by microscopic observation to confirm the absence of pathogen before stocking and the system at a density of 1 larva/L. The larvae were fed with pelleted feed of proper size range and the crude protein content of the feed was calculated by microkjeldhal method (APHA, 1998) as 30%. Detritus management was done using the probiotic Detrodigest and *Vibrio* population was controlled using an anti vibrio probiotic *Micrococcus* MCCB104 added into the system once in three days.

Two trails of recirculation experiments were carried out. In trial 1, the animals were fed in low quantity of feed starting from 1.75g/day and then the quantity slowly increased for a period of 70 days. The average body weight of the animals and the algal counts were determined every 10 days. Total suspend solids and

total dissolved solid were estimated (APHA, 1998) every 10 days to study the effect of addition of Detrodigest into the culture system. *Vibrio* count was taken every 10 days to assess the effect of probiotic *Micrococcus* MCCB104.

In the trial 2, the feeding rate was slightly higher starting from 60 g/day. The animals were reared for 60 days as in trial 1 and the average body weight and length were calculated every 7 days. In both the trials, TAN, NO_2-N and NO_3-N were estimated daily.

Since the system was in complete recirculating mode without water exchange, the source of TAN production in the system was assumed to be only through feed and excretion by the animals reared. The in situ nitrification was neglected. The total daily TAN production (P_{TAN}) based upon the fish feeding rate was therefore taken as the TAN into the system and calculated using the following equation (Timmons et al., 2001):

$$P_{TAN} = FA \times PC \times 0.092 / day \qquad \text{.....(5.2)}$$

where,

PTAN = Rate of ammonia production (g TAN/day)

FA = Amount of feed per feeding (g)

PC = Protein Content of the Feed (%)

0.092 is the fraction of protein nitrogen that is excreted as TAN.

The volumetric total ammonia nitrogen conversion rate (VTR) is used as the principal indicator for evaluation of the filter performance (Pfeiffer and Malone, 2006, Colt et al., 2006). The VTR was obtained by using

$$VTR = \frac{K_c (TAN_I - TAN_E) Q_R}{V_b} \qquad \text{.....(5.2)}$$

where,

VTR is the g TAN converted per m^3 of filter media per day

Q_R the flow rate through the filter (L/pm)

K_c the unit conversion factor of 1.44

TAN_I and TAN_E the influent and effluent ammonia (mg/L)

V_b is the volume of filter media (0.023 m^3).

The VTR for each TAN concentration was estimated and regression analysis of TAN versus VTR was carried out

The volumetric biomass capacity of the system was estimated by dividing the biomass (g) by V_{media} (m^3) (Colt et. al., 2006).

5.3 Results and discussions

5.3.1 Validation of stringed bed suspended bioreactors

5.3.1.1. Maintenance of *Penaeus monodon* adults and brooders

The maintenance of adults and brooders of *P. monodon* in experimental systems without water exchange even at laboratory level was an extremely difficult task as slight alternations in water quality used to lead to mortality. Integration of SBSBRs to the holding system showed that water quality could be maintained to the acceptable level in the rearing systems with out water exchange. During the trail average pH was in the range 7.6-7.8, salinity 15ppt and alkalinity 58.5 mg/L. TAN, nitrite and nitrate were within the acceptable levels (Fig. 2). The brooders also could be successfully maintained healthy for longer period with the help of the SBSBRs. The water quality in the brooder rearing system with 10 brooders of 150 g was with in the acceptable levels (Table 2, Fig. 3). Based on these results it has been inferred that SBSBRs can be used in live transportation of spawners and adults from the point of collection to the hatchery and for their quarantine till shifted to the maturation or breeding tank. It has to be pointed out that the reactors are sufficiently smaller enough to be incorporated in to the transportation containers.

5.3.1.2 *Macrobrachium rosenbergii* and *Penaeus monodon* seed production systems

The water quality parameters in the experimental and control tanks are summarized in Table 3. In both the systems, there were no significant differences in the parameters between the control and test tanks except the moderately higher total bacterial population recorded in the control tanks (Table 3). During

validation of the reactors, nitrification was found established in the experimental tanks instantly and progressed rapidly from the 3rd day onwards (Fig. 4 and 5). There was significant ammonia ($P<0.001$) and nitrite ($P<0.05$) removal in both prawn and shrimp larval production systems compared to that in the control, where ammonia oxidation was found set in only after 8 days of commencement of the experiment with no nitrite oxidation. Meanwhile in the larval rearing tanks with the reactors, NH_4^+-N and NO_2^--N were not found built up, instead, there was a steady increase of NO_3^--N. This proved establishment of the two step nitrification process in larval rearing tanks deployed with the reactors. During the experiment ammonia and nitrite concentrations in the control tanks were found gone up above 2 mg/L and 1.6 mg/L respectively whereas in the experimental tanks both were always below 0.25mg/L.

On terminating the experiment at PL-5 in the case of *M. rosenbergii* and at PL 15 in the case of *P. monodon*, the relative per cent survival and metamorphosis was found significantly higher ($P<0.01$) in the experimental tanks with the activated bioreactors (Table 4).

In biological ammonia removal systems nitrifying activity of suspended bacteria has been reported to be extremely low, due to slow growth rate and inhibition of nitrification by free ammonia and nitrite under the alkaline conditions of seawater (Bower and Turner, 1981; Furukwa et al., 1993). Without the addition of nitrifiers as start-up cultures, 2-3 months are needed to establish nitrification in marine systems (Manthe and Malone, 1987) and 2-3 weeks in freshwater (Masser et al., 1999). There is an agreement among researchers and between laboratory research and commercial applications on the fact that saltwater systems need a much longer start-up period. Under such situations, immobilization techniques have been found useful to overcome the delay in the initiation of nitrification (Sung-Koo et al., 2000). Integration of activated SBSBRs to prawn and shrimp hatcheries was found to be an important means of overcoming this difficulty

Maintenance of ammonia and nitrite during larval rearing is crucial as they cause lethal and sublethal toxicity and plays an important role in the production of healthy and properly sized fingerlings (Fielder and Allan, 1997). Marine larval systems can demand TAN and TNN levels below 1.0 mg/L well below the maximum set for the oligotrophic category (0.3 mg/L N) (Malone and Beecher, 2000). During the validation of the reactors, TAN and TNN could be maintained bellow 0.25 mg/L.

The SBSBRs described here are designed for setting up nitrification in shrimp/prawn larval rearing tanks during the operation. The technology is relatively user friendly in the sense that they can easily be lifted out of water during disinfection and also can be shifted from one rearing tank to another. Loss of beads encountered in many biofilter systems (Timmons et al., 2006) are not experienced in the case of SBSBRs as the beads are stringed together within the reactors. Moreover the operational costs of the reactors are minimal and no energy costs are added up to the overall production cost as the aeration system already available in the hatchery are used for operating the airlift pumps.

5.3.2 Validation of packed bed bioreactors

5.3.2.1 *Macrobrachium rosenbergii* seed production system

The minimum and maximum values of pH, temperature, salinity, alkalinity, hardness and total bacterial count in the rearing water of the experimental and control tanks during each treatment are summarized in Table 5. Heterotrophic bacterial community (CFU in ZoBell's Marine Agar) in the control tank increased substantially and there was no remarkable difference in the other water quality parameters between the tanks. The extent of nitrification during the period is presented in Fig. 6. In the control tanks TAN exhibited progressive increase with its subsequent decline and concomitant increase of NO_2-N after 14 days; however, NO_3-N was never found built up in the system. Meanwhile, there was significant TAN removal ($P<0.01$) in the experimental tanks with significant ($P<0.05$) NO_2-N removal. Within 8 days both TAN and NO_2-N concentrations were below

detectable levels. NO_3-N exhibited progressive increase to 7.6 mg/L within 17 days of the experiment.

M. rosenbergii is an inhabitant of fresh water, however, its larval stages are completed in saline waters with salinity 13-15 ppt and therefore, in the larval rearing systems bioreactors operating in this salinity regime are essential. In saltwater systems RAS plays an important role in the production of healthy and properly sized fingerlings (Fielder and Allan, 1997) and has significant implications as the system demands operation under oligotrophic conditions. In the bioreactor integrated larval rearing systems, ammonia oxidation was established within a day and it took eight days for nitrite oxidation whereas in the control 14 days were required for the initiation of nitrification. The delay in establishing active nitrite oxidation might be due to the lower multiplication rate of nitrite oxidizers compared to that of ammonia oxidizers (Paller 1992). The maximum average TAN and NO_2-N concentrations in the experimental larval rearing tanks were 0.18 and 0.25 mg/L respectively typical of any marine systems. Marine larval rearing systems demands TAN and TNN levels below 0.1 mg/L well below the maximum limit (0.3 mgN/L) under the oligotrophic category (Malone and Beecher 2000). During the progression of the experiment the NO_3-N concentrations increased up to 7.6 mg/L, however, remaining well below the toxic levels for *M. rosenbergii* larval culture (Mallasen et al., 2004). In an experimental study, significantly lower survival rates of *M. rosenbergii* larvae were noticed at total ammonia concentrations ranging from1 to 8 mg/ L at pH 9, whereas at pH 7 and 8 survival rates were high (Mallasen et al., 2004). In the present field evaluatiion carried out at higher stocking densities (200/L) at a salinty of 15ppt, there was significantly higher percent survival of larvae in the system integrated with the bioreactors when compared to the control. This shall enable hatchery systems to operate as closed recirculating systems.

The overall per cent survival of larvae in the control and test tanks was estimated and presented in Table 6. The tank with the reactor exhibited significantly higher

(P<0.001) percentage survival (LSD at 0.1% =15.19) with an average relative percentage survival (RPS) of 22. 86 %.

The average water quality parameters of the spent water are given in Table 7. TAN, NO_2- N and NO_3-N were lower in the experimental tanks than in those of the controls (4th day) indicating higher percentage removal of TAN (78%), NO_2-N (79%) and BOD (56%).

Under oligotrophic conditions ammonia diffuses into a relatively thin vertically homogenous biofilm that is dominated by autotrophs, principally due to low BOD (<5 g/m^3) of the culture water (Malone and DeLosReyes, 1997). In the present study organic loading to the system was as low as 0.31 mg/L BOD and there has been minimal heterotrophic inhibition of nitrification (Satoh et al., 2000; Zhu and Chen, 2001) evidenced by the progressive increase nitrification rates. On biosecurity perspective (Otoshi et al. 2003; Pruder, 2004) water recirculation dramatically reduces the possibility of pathogen introduction (Goldburg et al., 2001). In this context integration of packed bed bioreactor for nitrification of hatchery spent water with high percentage removal of ammonia (78%), and nitrite (79%) by 4th day strengthens the possibility of reuse of water with limited discharge and reduced intake paving the way for bio-security.

5.3.2.2 *Penaeus monodon* maturation system

Upon integrating the PBBRs into the maturation system a remarkable reduction in the total ammonia concentration was observed within a week. The total ammonia concentration in the maturation tank was 4.47 ppm during the day of integration of the reactors, within a week this came down to 0.122 ppm, showing the rapid setting up of nitrification (Fig.7). Nitrite also showed a reduction after a slight increase initially. Nitrate concentrations were always bellow 5 mg/L. Bacterial population enumerated on TCBS from the water samples were 30 CFU/mL during the initial days of experiment which gradually declined to zero at the end of the experimental period. Recirculating systems for captive maturation and reproduction of penaeids remain relatively primitive. They depend upon a delicate

balance of environmental parameters, eyestalk ablation and a relatively frequent replacement of broodstock and a diet of expensive fresh frozen feeds to maintain nauplii production levels. In the hatchery, problems with unexplained mortality and the presence of pathogenic bacteria continue to affect the stability of production and seed quality (Browdy, 1998). However, in the present experiments with the integration of PBBR the water quality could be maintained at the desired levels.

5.3.3. Laboratory level recirculating aquaculture system for *Penaeus monodon*

The progress of nitrification in trial 1 with increasing feed rate showed that (Fig.8) TAN, NO_3-N and NO_2-N were within acceptable levels. The TDS and TSS (Fig.9) could be maintained in the rearing water during the progression of culture with the addition of Detordigest whereas *Vibrio* population in the system could be maintained by the addition of *Micrococcus* (Fig.10). There was sustained algal production and the animals could gain a weight of 9.4 g in 70 days (Fig.11). The VTR of the reactors increased with increasing TAN concentration (Fig. 12). In trial 1 the average VTR for the largest feed rate of 150 gm was 0.1361±0.0083 kgTAN/m^3/day. In trial 2, the average VTR during the highest feeding rate of 160 gm from 54-60th days of culture was 0.1533±0.0045 kgTAN/m^3/day. The regression between VTR and TAN explained 98% variability in VTR ($P<0.001$) (Fig 13). In trial 2 also TAN, NO_3-N and NO_2-N were within acceptable levels (Fig.14). The regression between VTR and TAN explained 86% variability in VTR ($P<0.001$) (Fig 15).

Numerous studies have indicated that increasing the TAN concentration in biofilters results in proportional improvements in a filter's conversion ability (Rogers and Klemetson, 1985; De Los Reyes and Lawson, 1996; Malone et al., 1999; Sandu et al., 2002). In both trials the application of probitocs was successful in breaking down the feed residues and keeping the *Vibrio* population stable.

The volumetric TAN conversion rate (VTR, kg TAN/ m^3) of biofilters commonly used in RAS are: floating bead filter, 0.07-0.35; fluidized sand filter, 0.1-2.7; trickling filter, 0.02-0.64; moving bed filter, 0.51-2.22; RBC, 0.10-0.13; and submerged filter, 0.01. Backwash frequency has a significant effect on the bead filter nitrification rate and Golz et al. (1999) determined that a high VTR (kg TAN/ m^3/ d) of 0.37 could be achieved by a bubble-washed bead filter with an 8 hr backwash interval and an optimal VTR (kg TAN/ m^3/ d) of 0.39 for an aggressively-washed bead filter at a 48 hr backwash interval. Recommended nitrification rates for fluidized bed filters were 0.7 (kg TAN/ m^3/ d)for applications in cold water systems and 1.0 (kg TAN/ m^3/ d) for warm water systems based on a series of pilot scale tests (Timmons et al., 2001). However, the nitrification performance of a commercial fluidized sand filter system reported much lower nitrification rates with 0.35-0.49 (kg TAN/ m^3/ d) in a cold water system (Summerfelt et al., 2004) and 0.1 (kg TAN/ m^3/ d) in a warm water system (Pfeiffer and Malone, 2006). The values obtained in the present study are comparable to values suggested by Malone and Beecher (2000). Based on over ten years of floating bead filter research, they (Malone et al., 1998; Malone and Beecher, 2000) recommended the use of a VTR (kg TAN/ m^3/ d)of 0.035-0.105, 0.07-0.18, and 0.14-0.35, for the design of floating bead filters in brood stock, ornamental, and growout systems, respectively, for warm water systems.

The average biomass yield was 9.4 at the end of 70 days culture in trail 1 (Fig 11) and 10.3 g (Fig. 16) in trial 2 at the end of 60 day culture with 60% survival. The mortality was attributed to cannibalism. The maximum volumetric biomass capacity of the system was 68 g/m^3 in trial 1 and 75 g/m^3 in trial 2. This is only a preliminary study showing the effective usage of bioreactors and probiotics for maintaining a suitable water quality for the culturing of *P. monodon* .To make this process completely organic we have to supply organically produced feed also. The main problem observed during the culture period was the cannabalisam, so the formulation of the diet is a critical component in the recirculating organic culture. The Stringed Bed Suspended Bioreactors an Packed Bed Bioreactors developed can form integral

parts of Organic Shrimp and Prawn Seed Production system as they are the systems specially designed for tropics using indigenous nitrifying bacterial consortia. Currently organic standards are manly oriented towards temperate species and it is required to develop organic systems for tropical regions (Chen, 2004). This technology is now commercialized through M/S Oriental Aquamarine Biotech India Pvt Ltd, U-140, Kovaipudur, Coimbatore-641 042

Based on the experimental results a novel Recirculation System for Organic Shrimp and Prawn Seed Production could be designed, principally by integrating the reactors at three locations in a larval production system such as, 1. PBBRs in the reservoir system. 2. PBBRs at brood stock maturation system and 3. SBSBRs at the larval rearing tanks. The probiotic Detrodigest can be used for the management of detritus in the systems. This organism, in association with the heterotrophic bacteria in the nitrifying bacterial consortia, rapidly digests the detritus into monomeric substances and helps attain reef quality water. For the prevention of entry of *Vibrio* sp. through brood stock and nauplii, *Micrococcus* MCCB104 could be used. The gut probiotic (Enterotrophotic- 1) is supplied in all compartments of the larval rearing system such as algal production system, *Artemia* production unit, larval rearing tank and maturation system so as to prevent colonization of the pathogenic vibrios any were in the production facility (Rejish Kumar et al., 2006).

Quantifiable advantages of implementing organic seed production systems are 1. Increase in out put of seed by not less than 20% from the existing level 2. Better performance of seed under field conditions 3. Avoidance of frequent pumping of water from sea and disinfection as the same water is recirculated and reused 4. Decrease in recurring cost and increase in profits as the cumulative effect and 5. Stabilization of maturation system and self-sufficiency in nauplii production

To sustain the shrimp industry in India, the production process has to match with the global standards and in this requirement the importance of organically produced good quality seed need not require any more emphasis. The

technology package presented here is first of its kind globally and is the one developed indigenously. Adoption of the technology in Indian shrimp/ prawn production process is the first step towards the beginning of organic shrimp farming in India.

Table 2. Water and microbial quality of the rearing tank during the maintenance *Penaeus monodon* brooder in laboratory rearing systems integrated with SBSBR

pH	8-7.7
Salinity (ppt)	32
Temperature (° C)	27-29
Alkalinity (mg of $CaCO_3$/L)	72-69
Hardness (mg of $CaCO_3$/L)	5820
TPC (CFU/mL)	1.43×10^6 to 1.82×10^8
Vibrio count (CFU/mL)	1000-75

Table 3. Physico-chemical and microbial quality of rearing water in hatchery systems integrated with SBSBR during the experiment (n=3)

Rearing water quality	*P.monodon* system		*M.rosenbergii* system	
	Test tank	Control tank	Test tank	Control tank
pH	7.5-8	7.5-8	7.5-8	7.5-8
Salinity (ppt)	30-32	30-32	14-16	14-16
Temperature(° C)	27-29	27-29	28-31	28-31
Alkalinity (mg of $CaCO_3$ /L)	60-69	72-78	62-70	65-70
Hardness (mg of $CaCO_3$ /L)	5790-5820	5790-5950	2190-2260	2100-2277
TPC (CFU/mL)	1.43×10^6 to 1.82×10^8	1.59×10^6 to 3.78×10^9	4.51×10^5 to 2.27×10^7	1.73×10^5 to 1.03×10^9

Table 4. Impact of SBSBR in larval survival and metamorphosis on integration in to hatchery systems (n=3)

Hatchery system	Treatment	Survival (%)	P	Relative survival (%)
P. monodon	Control tank	16.33±1.53	<0.01	17.67
	Test tank	31± 1.73		
M. rosenbergii	Control tank	19.67± 1.53	<0.01	20.67
	Test tank	36.33±3.51		

Table 5. Physico-chemical and microbial quality of rearing water in control and test tanks with PBBR in *Macrobrachium rosenbergii* hatchery during the experiment (n=3)

Water quality parameters	Control tank	Test tank
pH	7.5-8.0	7.5-8.0
Salinity (ppt)	14-15	14-15
Temperature(°C)	28-31	28-31
Alkalinity (mg of $CaCO_3$/L)	66-70	64-70
Hardness (mg of $CaCO_3$/L)	2987-2900	2876-2900
TPC (CFU/mL)	1.91 x 10^5 to 1.14x 10^9	2.51x 10^5 to 4.21x 10^7

Table 6 Larval survival after the integration of PBBR into the *Macrobrachium rosenbergii* hatchery system

Treatment	% Survival	Average % Survival	Relative % Survival	ANOVA			
				Source of variation	df	Mean sum of squares	P
Control tank	a 18	18.33±1.	22.86	Between	1	522.67	<0.001
	b 17	53		treatments			
	c 20						
Test tank	a 36	37±2.65		Within	4	4.67	
	b 35			treatment			
	c 40						

Table 7. Mean water quality parameters of the spent water from *Macrobrachium rosenbergii* hatchery during the experiment (N=3).

Parameter	Control Tank	Test tank
Salinity (ppt)	15.33±0.47	15±0.41
pH	8.09±0.07	8.36±0.12
Eh (mV)	110±0.82	104.33±16.46
Ammonia (mg/L)	3.71±0.43	0.83±1.46
Nitrite (mg/L)	2±0.23	0.43±0.55
Nitrate (mg/L))	0.3±0.09	13.28±6.57
Phosphate (mg/L)	0.03±0.04	0.02±0.01
Sulphate (mg/L))	15.93±1.23	13.35±0.37
Alkalinity (mg $CaCO_3$/L)	79.33±0.94	79.83±5.34
Hardness (mg $CaCO_3$/L)	2483.33±107.81	2672.83±134.64
Chloride (mg/L)	10273.513±295.31	10505.83±504.00
DO (mg/LO_2)	5.73±0.39	5.81±0.64
BOD (mg/LO_2)	0.71±0.018	0.31±0.29

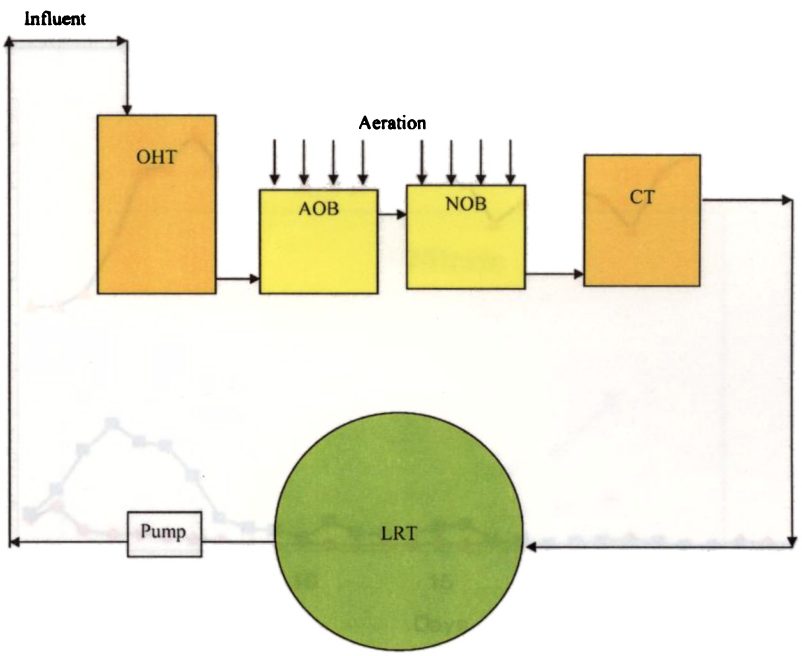


Fig. 1. Process flow diagram of the reactors integrated into the larval rearing tank (OHT-overhead tank with automatic water level controller, AOB-ammonia oxidizing bioreactor, NOB-nitrite oxidizing bioreactor, CT-collection tank for the treated water, LRT- larval rearing tank)

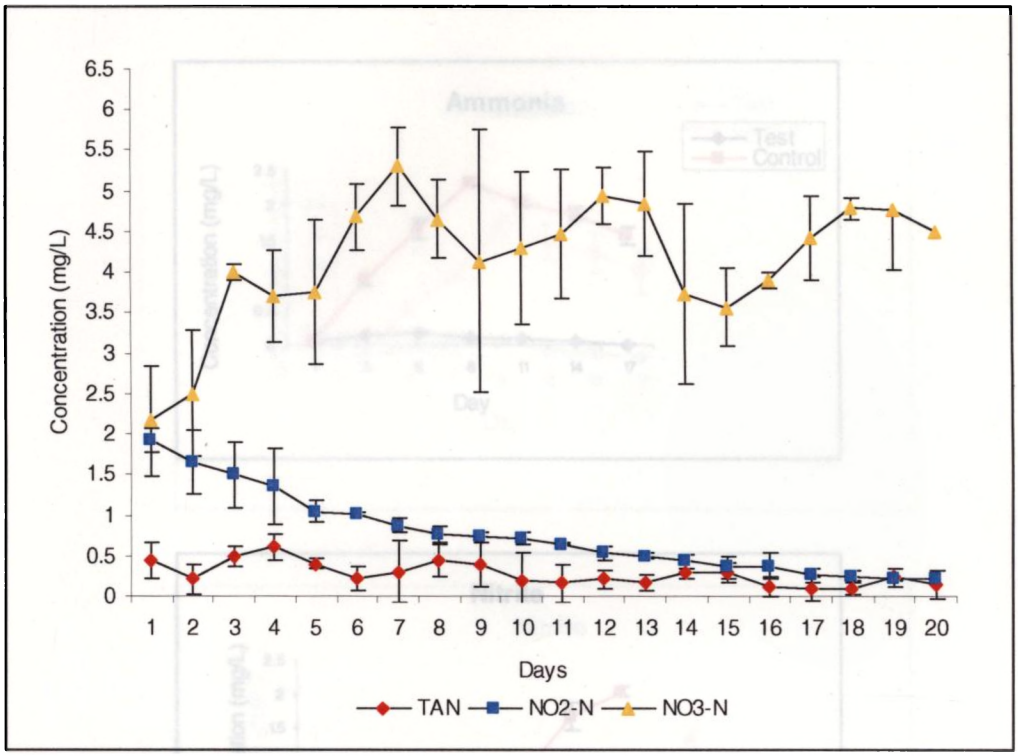


Fig.2. TAN, NO_2-N and NO_3-N in experimental systems integrated with SBSBR rearing *Penaeus monodon* adults

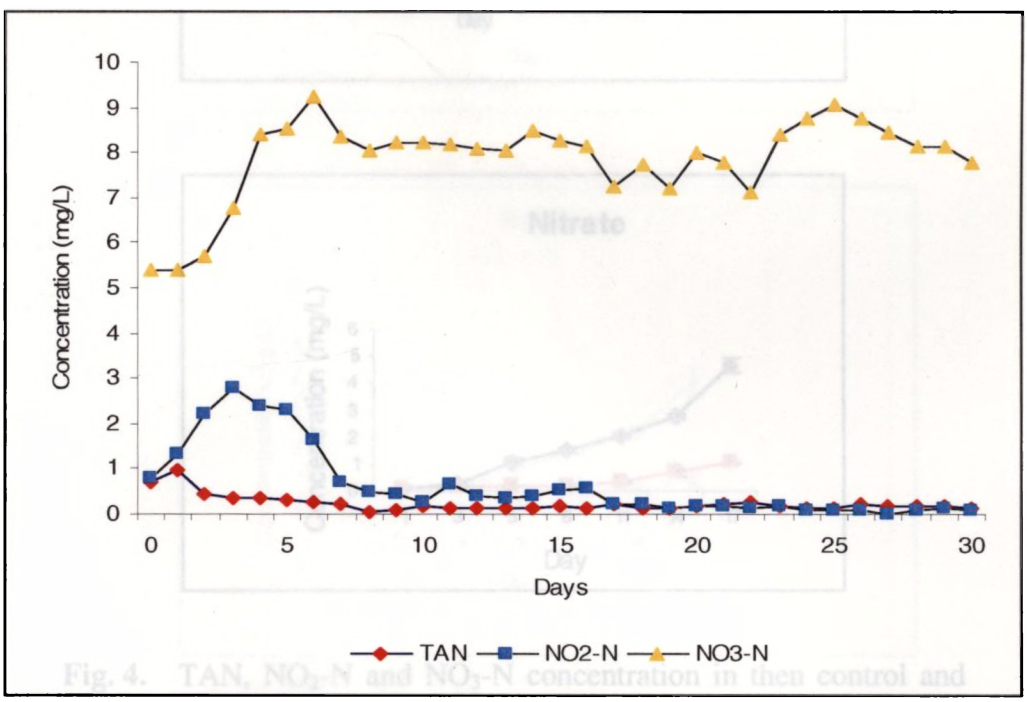
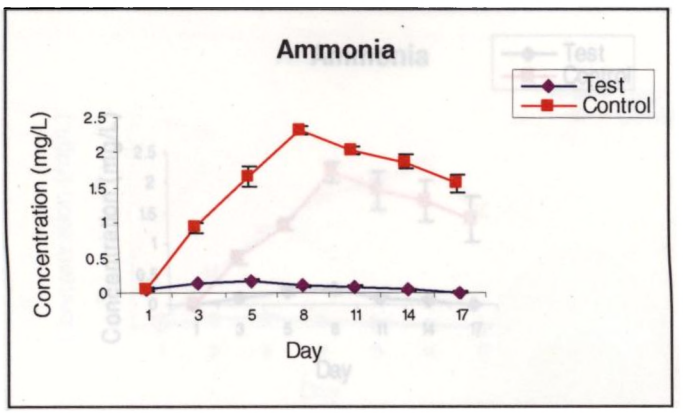
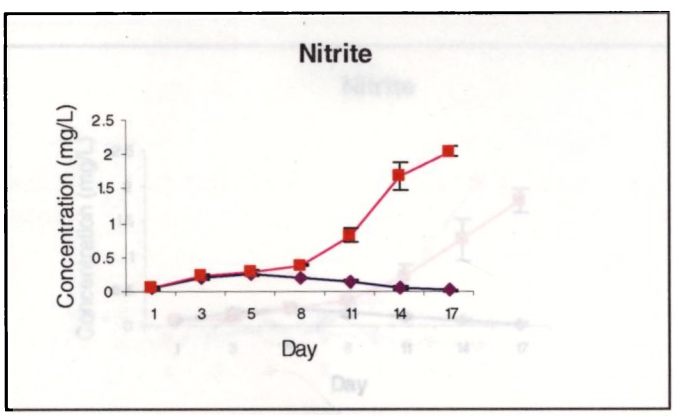


Fig.3. TAN, NO_2-N and NO3-N experimental systems integrated with SBSBR rearing *Penaeus monodon* brooders





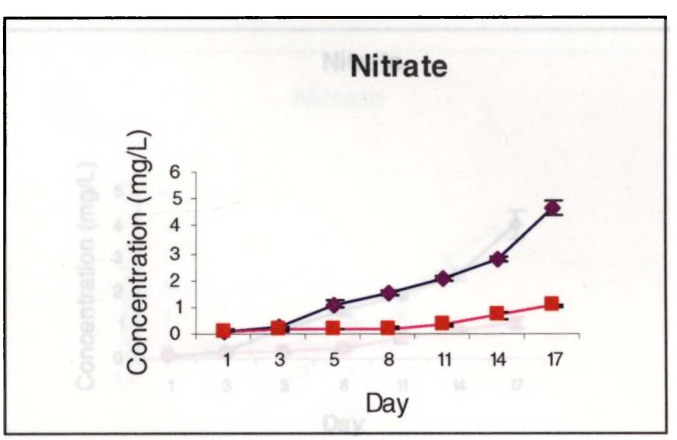


Fig. 4. TAN, NO_2-N and NO_3-N concentration in then control and SBSBR integrated *Macrobrachium rosenbergii* hatchery systems

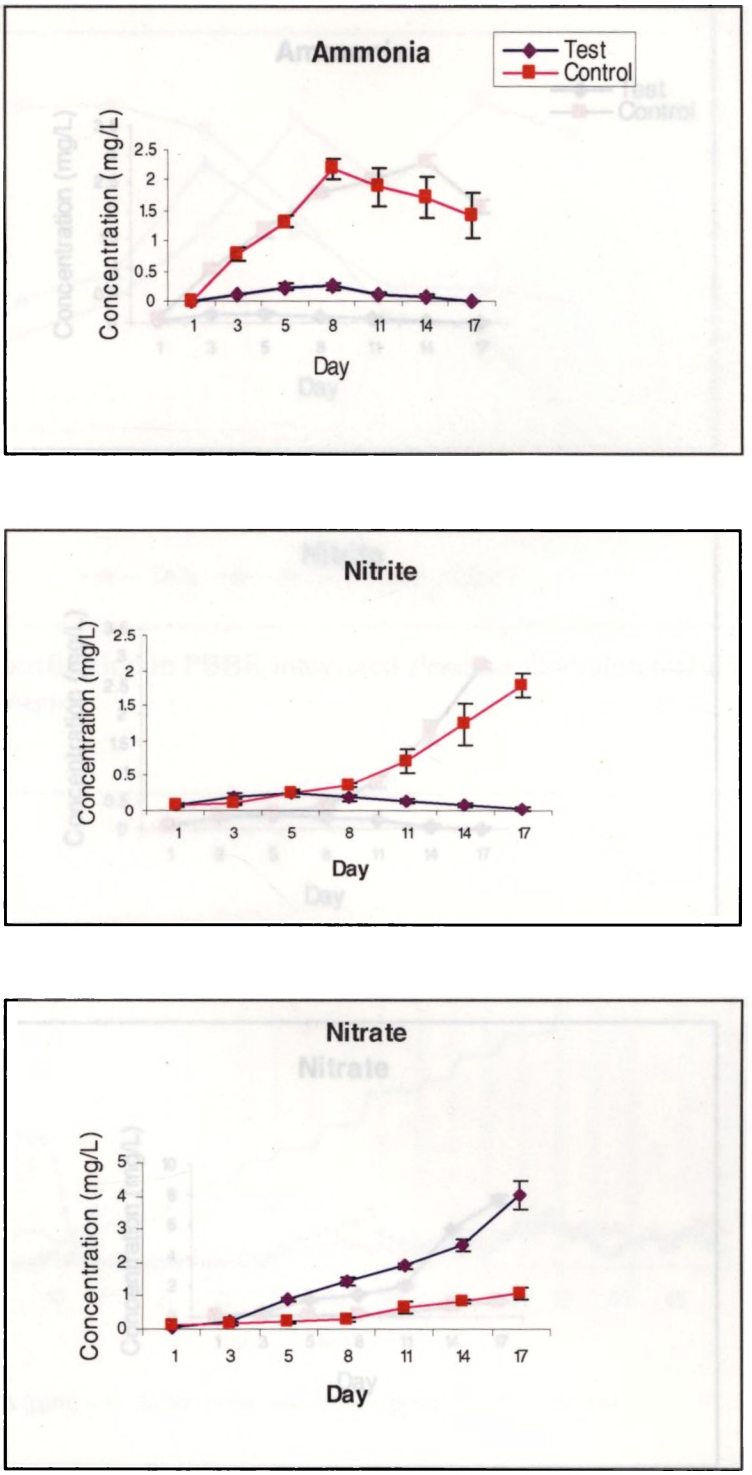


Fig.5. TAN, NO_3-N and NO_2-N concentrations in then control and SBSBR integrated *Penaeus monodon* hatchery systems

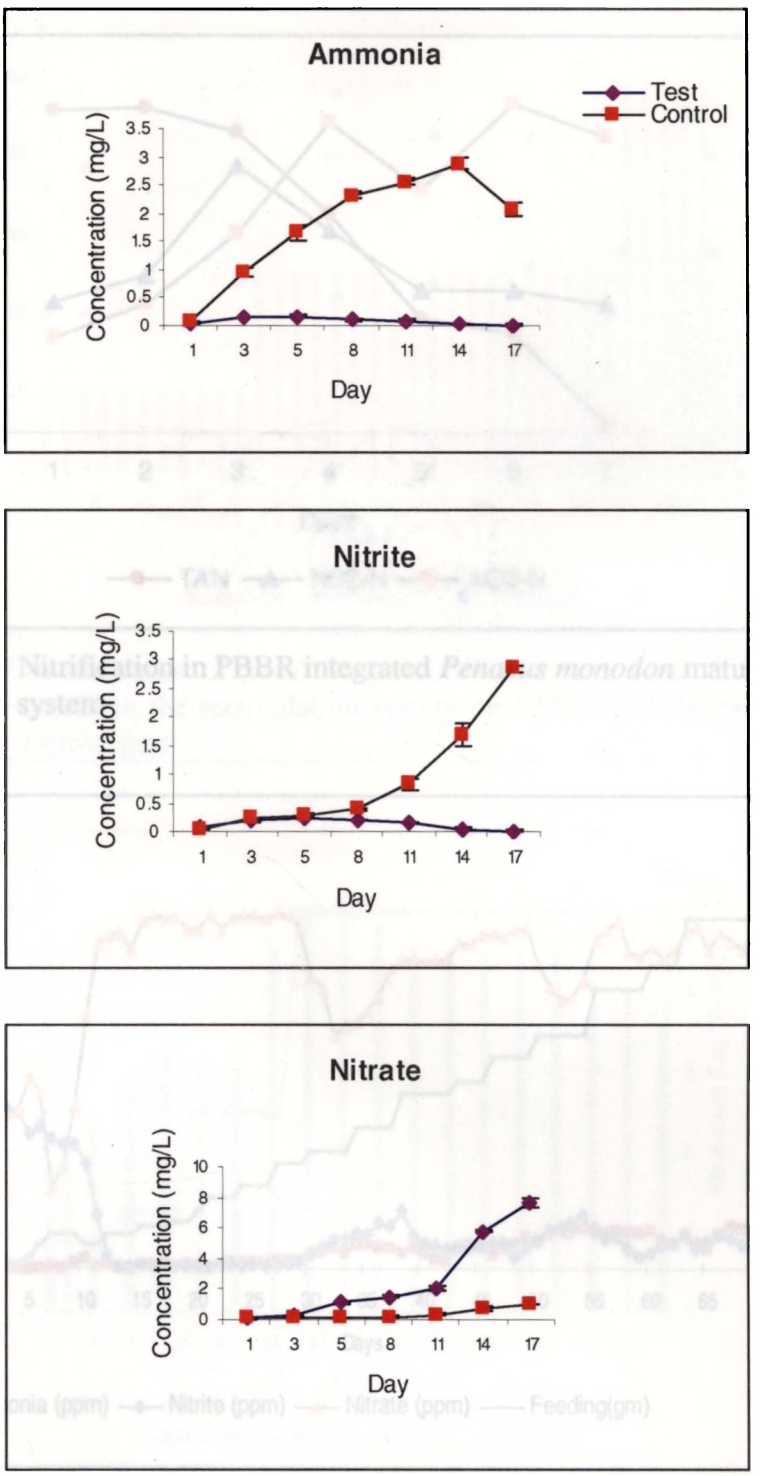


Fig. 6 Nitrification in the control and PBBR integrated *M. rosenbergii* hatchery systems (N=3)

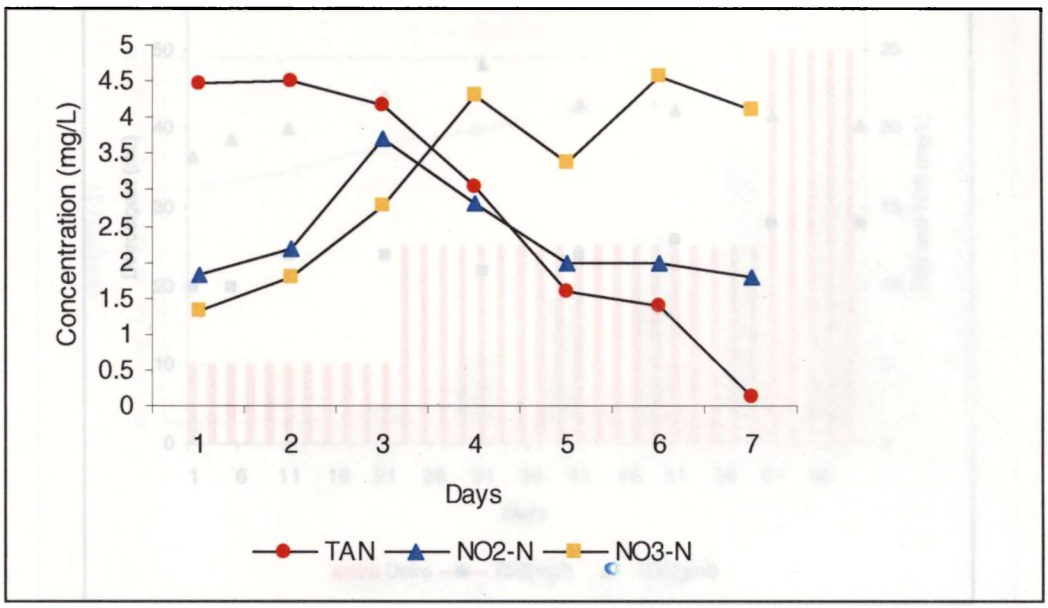


Fig. 7 Nitrification in PBBR integrated *Penaeus monodon* maturation system

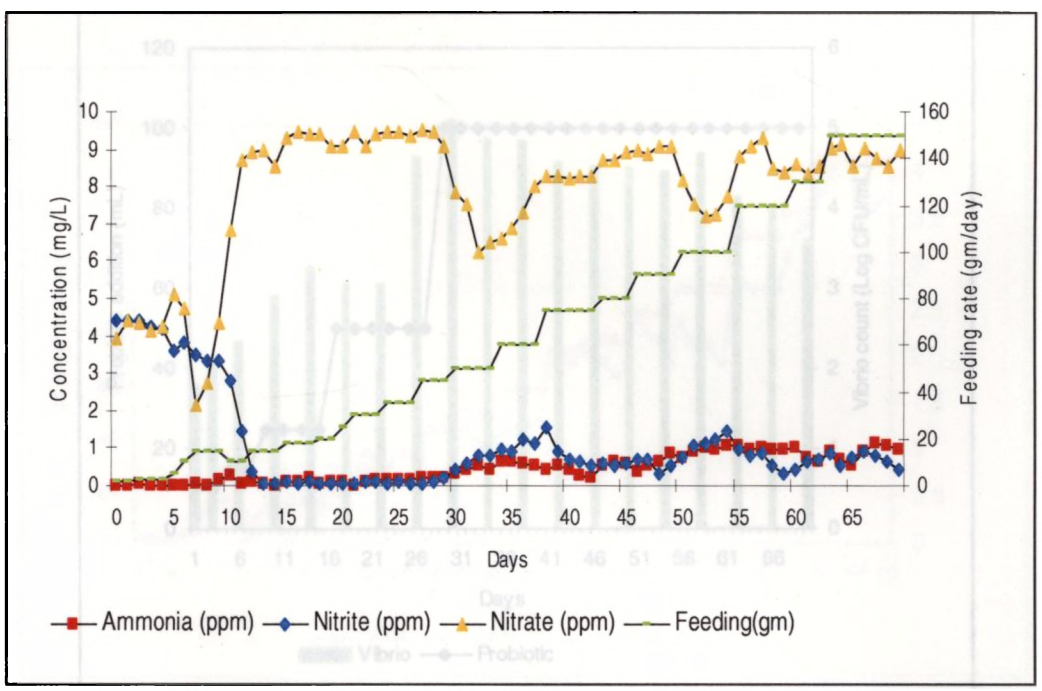


Fig.8. Progress of nitrification in recirculatory *Penaeus monodon* culture system (Trial 1)

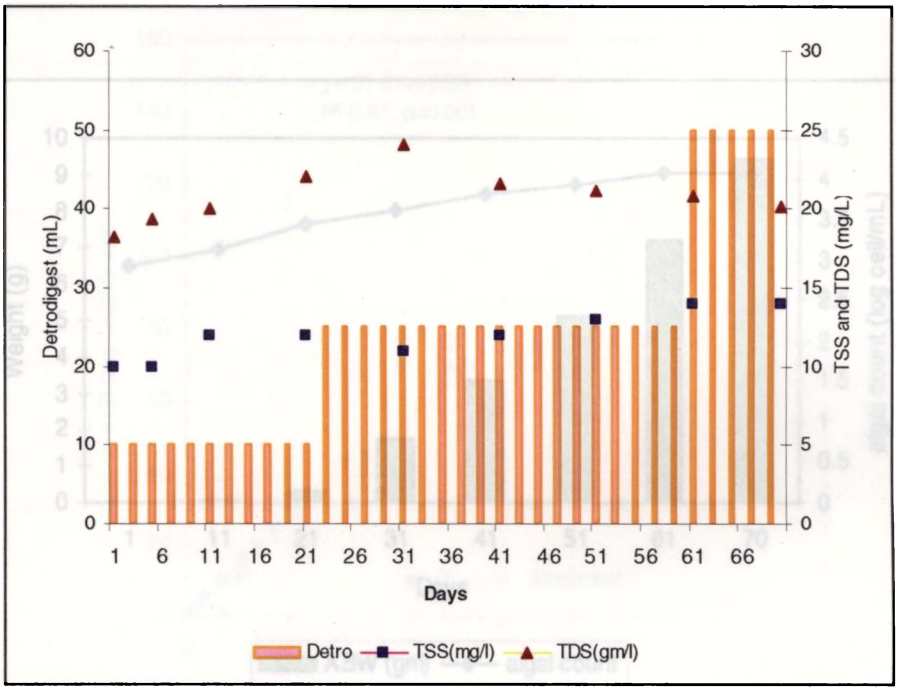


Fig. 9 Maintenance of Total Suspended Solids and Total Dissolved Solids in the recirculation system on addition of the probiotic Detrodigest

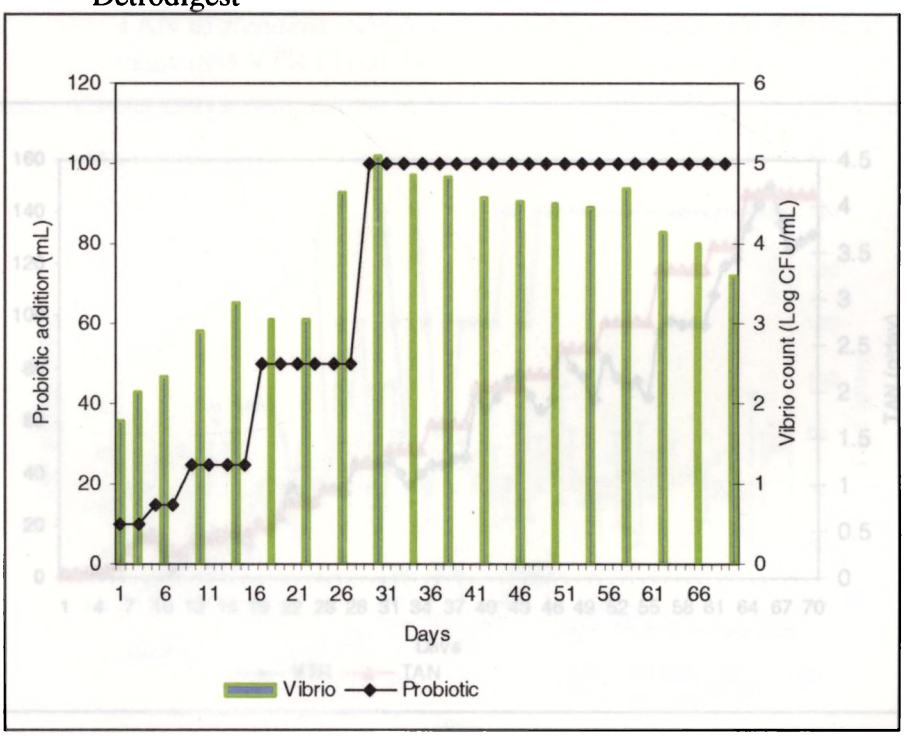


Fig. 10 Maintenance of *Vibrio* population in the recirculation system on addition of the probiotic *Micrococcus* MCCB104

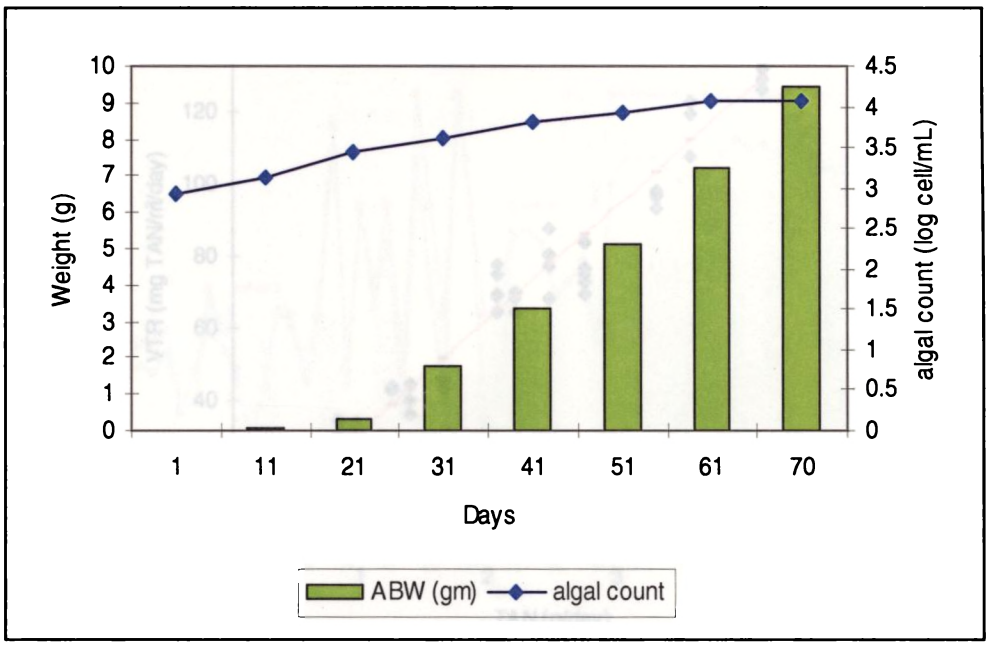


Fig. 11 Average body weight, algal count in *Penaeus mondon* recirculation system (Trial 1)

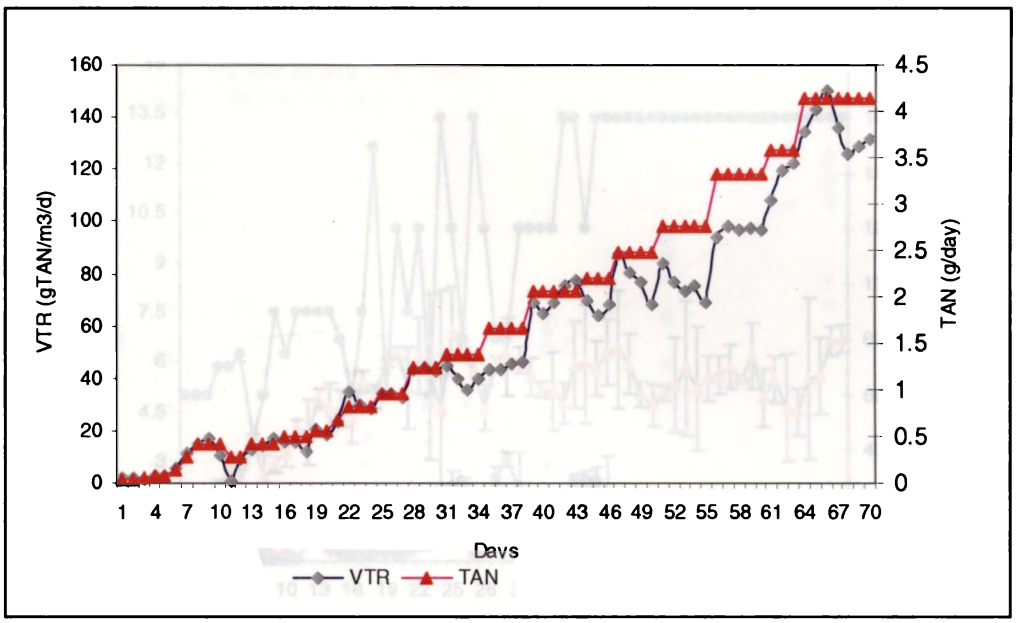


Fig. 12. Volumentric TAN Removal rates and TAN in *P. monodon* recirculation system (Trial 1)

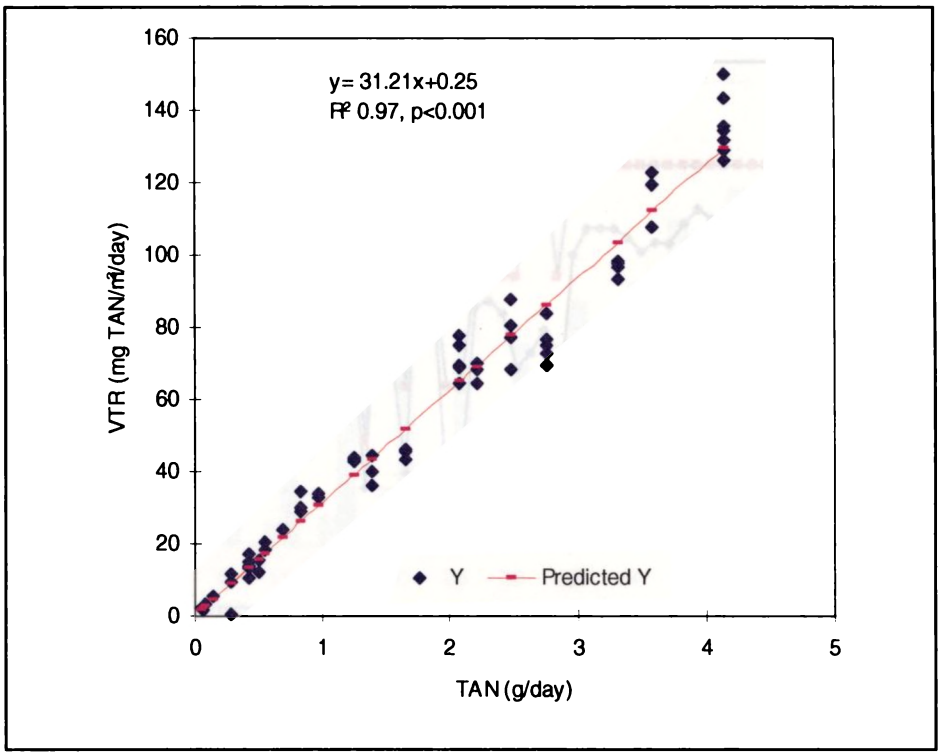


Fig. 13. Regression of Volumetric TAN Removal rates (VTR) versus TAN in *Penaeus mondon* recirculation system- Predicted and measured VTR (Trial 1)

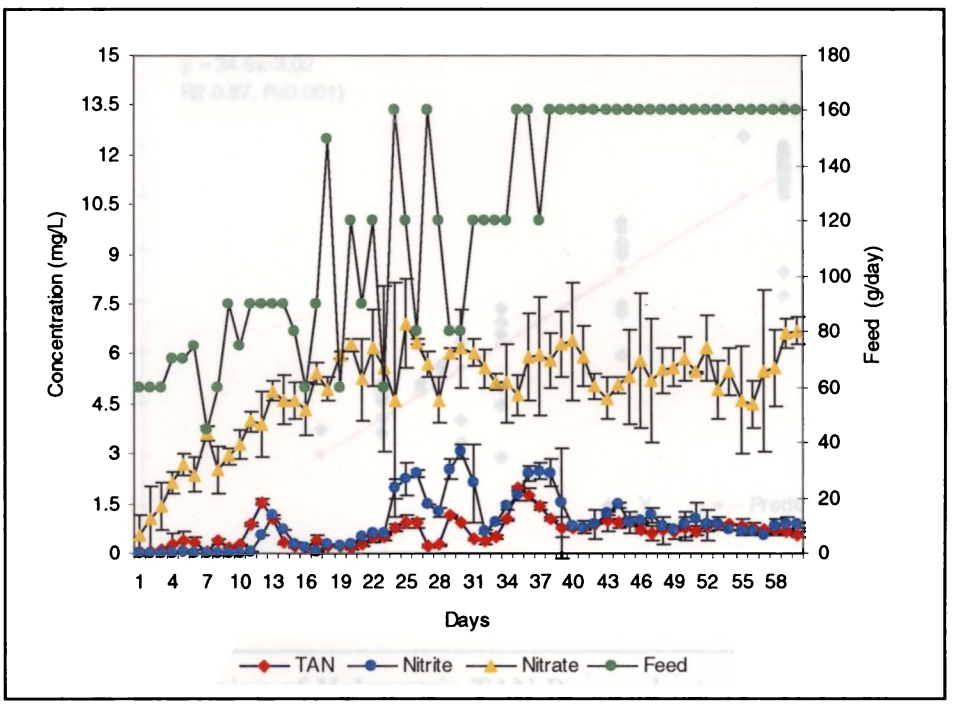


Fig.14. Progress of nitrification in recirculatory *Penaeus monodon* culture system (Trial 2)

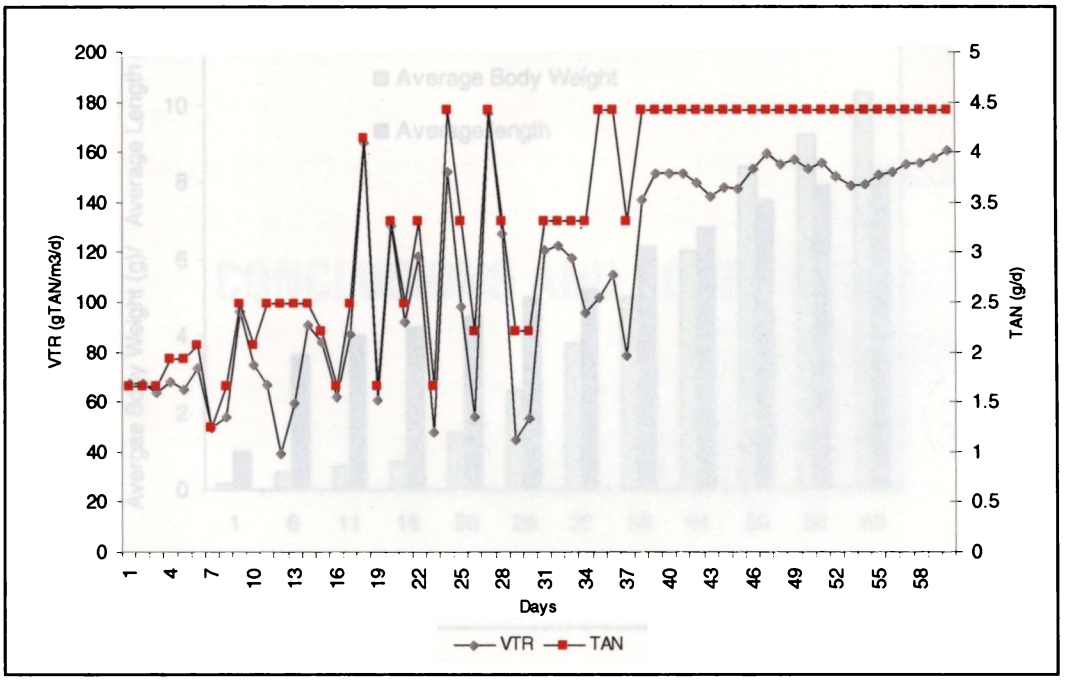


Fig. 15 Volumetric TAN Removal rates and TAN in *Penaeus monodon* recirculation system (Trial 2)

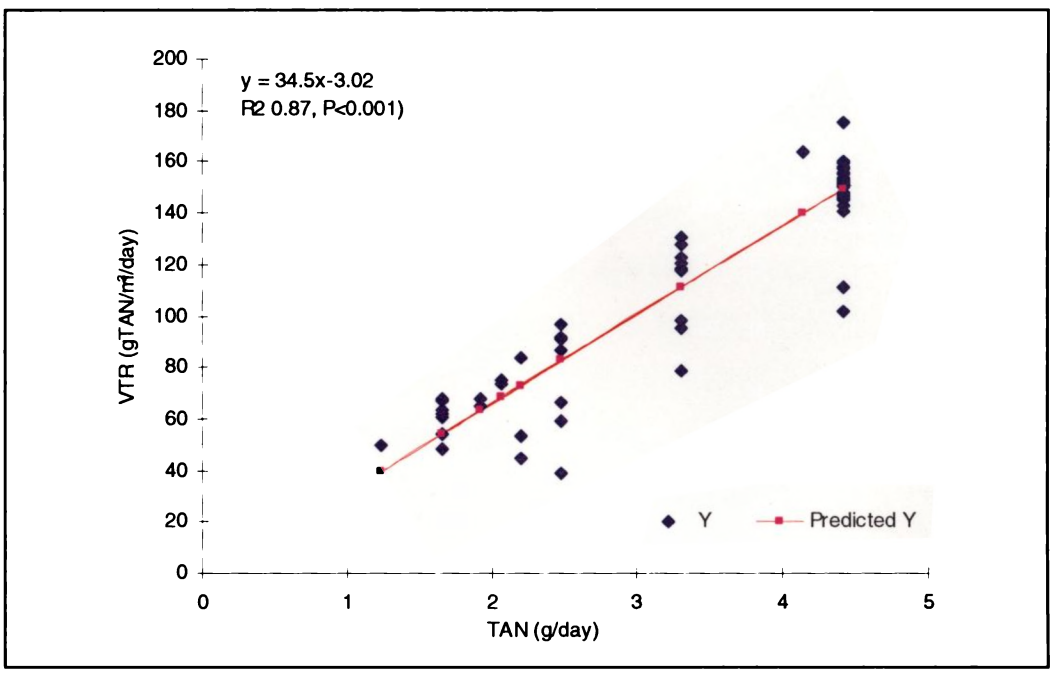


Fig. 16. Regression of Volumetric TAN Removal rates (VTR) versus TAN in *P. monodon* recirculation system- Predicted and measured VTR (Trial 2)

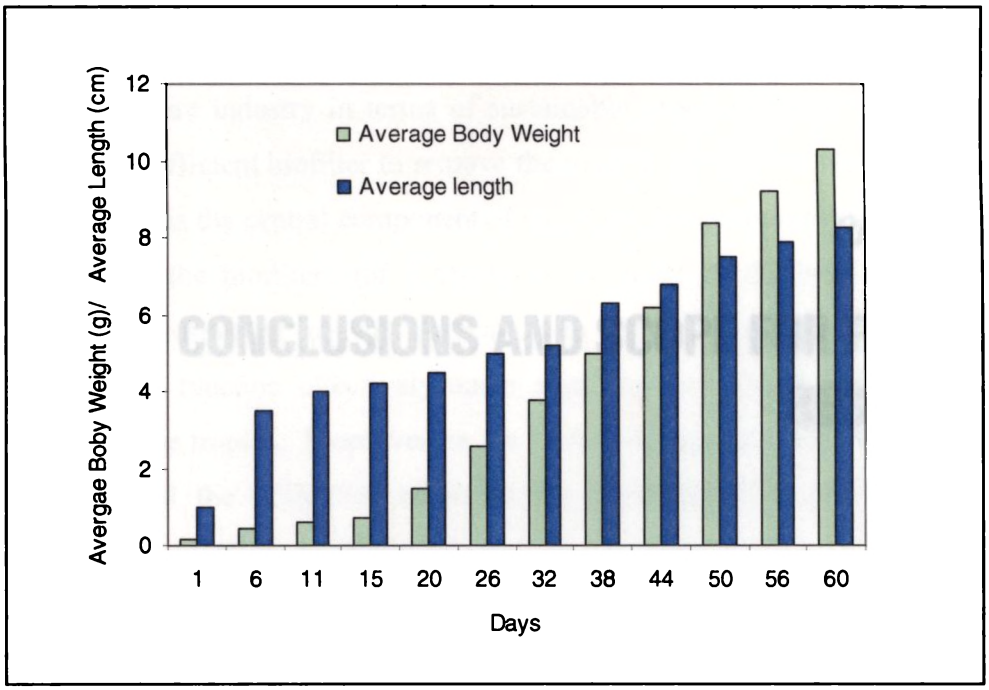


Fig. 17. Average body weight and length in *Penaeus monodon* recirculation system (Trial 2)

Chapter 6

CONCLUSIONS AND SCOPE FOR FUTURE RESEARCH

Recircualting aquaculture systems are gaining increasing demand in the global aquaculture industry in terms of sustainable production and environment protection. An efficient biofilter to remove the toxic ammonia and nitrite from the culture systems is the central component of any RAS. Extensive research has been carried out on the biofilters for RAS, however, most of the biofilter systems developed and validated are for application in temperate aquaculture systems, which may not function effectively under significantly different environmental conditions of the tropics. Moreover, in the tropics aquaculture is a livelihood for the people, and the design of cost-effective bioflilters is a more pragmatic approach than adopting existing technologies from the market. Taking these factors into consideration an *ex situ* packed bed bioreactor (PBBR) for the reuse and recycling of spent water for larval production, maturation systems and for the treatment of fresh seawater and an *in situ* Stringed Bed Suspended Bioreactor (SBSBR) for in situ nitrification especially in the larval rearing tanks were developed (Achuthan, 2000 and Anon, 2002). These bioreactors contain specially designed polystyrene and polyethelyene beads as the substrata for immobilizing nitrifying bacterial consortia. Two ammonia oxidizing consortia such as AMOPCU-1 (Ammonia oxidizers for penaeid culture)and AMONPCU-1 (Ammonia oxidizers for non-penaeid culture) and two nitrite oxidizing consortia such as NIOPCU-1 (Nitrite oxidizers for penaeid culture) and NIONPCU-1 (Nitrite oxidizers for non-penaeid culture) developed under constant salinity regimes from brackish water environments (Achuthan, 2000) were used for activating the reactors. For wide adoption of the technology in the market, it required further optimization, fine tuning and validation at pilot to field levels; the consortia required characterization for resolution of community and the present study is focused in this direction.

Characterization of the nitrifying bacterial consortia

Electron microscopy revealed characteristic nitrifying bacterial community in the consortium having the characteristic features such as cyst formation, presence of

intracytomembranes, carboxysomes and/or poly phosphate like inclusions, and the embedded nature of the cells in glycocalyx, matching with the previous reports. Fluorescence in situ hybridization analysis of the four consortia with one general bacteria probe and the other seven nitrifying bacterial specific probe confirmed the presence and diversity of autotrophic nitrifiers in the consortia. The groups detected were β- ammonia oxidizers (*Nitrosococcus mobilis, Nitrosospira*) and nitrite oxidizers (*Nitrobacter, Nitrospira*). However, 30 ppt nitrite oxidizers were found devoid of any ammonia oxidizers targeted by the selected probes.

As part of characterization, amplification and sequence analysis of the phylogenic gene 16S rRNA were accomplished using three sets of primers such a) Universal primer set (Reddy et al., 2001) which yielded a consistent specific 1500bp amplicon; b) primer set of Gauger and Gomez-Chiarri (2002) which yielded an amplicon of 800 bp in all the consortia exempt in NIONPCU-1 and c) primer set Nit A and Nit B specific for β- ammonia oxidizers.

Amplified Ribosomal DNA restriction Analysis (ARDRA) pointed out that there was not much variation in the community structure between the four consortia.

On matching the 16S rRNA gene sequences obtained with the clones of 15N cultures with the GenBank database using the BLAST algorithm the majority of the hits were with the sequences of uncultured bacteria and other heterotrophs like *Flavobacterium* sp., *F. mizutaii*, Bacteroidetes *bacterium* and uncultured *Sphingobacterium* sp.,while that of 30N clones blast with *Flexibacter tractuosus*, *Microscilla sericea*, *Alcanivorax dieselolei*, *Aleanivorax* sp., *Uruburuia balearica*, Uncultured *Cytophagales* and *Sphingobacteriales* bacterium. However, on comparing these sequences phylogenetically, significant similarities were lacking between these sequences.

The functional gene of ammonia oxidizing bacteria *amo*A could be amplified from both the ammonia oxidizing consortia. A sequence analysis showed that the

sequences matched with the existing *amo*A sequences of *Nitrosomonas* sp. and *Nitorsomonas europea.*

A significant observation is that the *amo*A genes could be amplified from ammonia oxidizing consortia alone and not from nitrite oxidizing consortia suggesting the soundness of functional gene approach.

An interesting feature of the consortia is the amplification of nitrite reductase gene, *nir* S with AMOPCU-1 and nonspecific amplifications with other consortia, indicating the denitrification potency of the consortia.

Eventhough several studies elsewhere have indicated the presence of archaeal nitrifiers in natural samples, no amplification could be obtained with archaeal primers with the four consortia investigated here.

In short ultrastructural similarity of the consortia with the classical nitrifiers, detection of β- ammonia oxidizers (*Nitrosococcus mobilis, Nitrosospira*) and nitrite oxidizers (*Nitrobacter, Nitrospira)* using FISH probes, amplification of amoA genes, no match of 16S rRNA gene with that of classical nitrifiers but their closeness with heterotrophs and amplification of nitrate reductase gene all make the consortia an interesting subject for further studies employing more robust molecular tools.

Development of mass production technique for the nitrifying bacterial consortia

The mass production of nitrifying bacterial cultures has been challenging owing to their slow growth rates and low growth yield. However, during the process of mass production only 4 to 6 days were required for activation, 20 to 25 days for amplification. In both these processes the quantity of the product formed was considerably higher than the substrate consumed as observed in our previous studies with nitrifying bacterial consortia generated from other sources. The maximum specific growth rate and yield coefficients of the consortia were higher

than the reported values for pure cultures of nitrifers. The maximum specific growth rate of the ammonia oxidizers at AMONPCU-1 (30ppt) and AMOPCU-1 (15 ppt) were 0.112 and 0.105/ hr whereas those for NIONPCU-1 (30ppt) and NIOPCU-1 (15ppt) were 0.11 and 0.123/ hr respectively. The indigenous nitrifying bacterial consortia production unit (NBCPU) designed and fabricated was successfully run in mass production of ammonia oxidizing consortia at 30 and 15 ppt salinities (AMOPCU-1 and AMONPCU-1) with both the consortia establishing a two step nitrification process.

There are reports that at field level the maximum nitrification capacity of salt water systems was considerably lower than in freshwater systems. The results showed that there was no delay in the initiation of nitrification during the mass production which is typical of marine systems. During this production process the cultures consumed comparatively lesser quantity of Na_2CO_3 for compensating the alkalinity destruction adding little to the cost of production. The biomass yield was higher in all the consortia. The higher yield coefficients suggested better energy conversion efficiency and higher CO_2 fixation potential of the consortia. The mass production process yielded high active biomass for immobilization in the bioreactors.

Nitrifying bioreactors for aquaculture systems: design specialties, activation, kinetics and modeling

Biofilter selection influences capital and operating costs of recirculating aquaculture systems, their water quality, and even the consistency of water treatment. The PBBRs were designed in such a way that the flow of water in the reactor system was maintained by gravitational force and energy required for the reactor operation could be restricted to pumping water to the reservoir tank and to operate an air pump to effect aeration. The system can be upgraded with filters and UV disinfection equipments. In case of incomplete nitrification after a single circulation, there is provision to recirculate it through the treatment system over and again. The SBSBRs are user friendly in the sense that they can easily be lifted

out of water during disinfection and also can be shifted from one rearing tank to another. The operational costs of the SBSBRs are minimal and no energy costs are added up to the overall production cost as the aeration system already available in the hatchery are used for operating the airlift pumps. SBSBRs can also find application during the live transportation of spawners. Both the reactors can be modified based on TAN load into the system by increasing the biomass of the nitrifying consortia used for activation of the reactors or by enhancing the hydraulic retention time or by increasing the number of reactors avoiding the over sizing or under sizing of the bioreactors, which is otherwise a commonly encountered practical problem. They are also interchangeable between prawn (salinity 15ppt) and shrimp (salinity 30 ppt) larval rearing.

During the activation of the bioreactors, the nitrification could be set within 24 hr in both bioreactors and in seven days the biomass immobilized into the filter media. The reactors were highly aerated systems and therefore, dissolved oxygen was never limited and always maintained at 5-6 mg/L. In PBBR the increasing flow rate was found to increase the TAN removal rate flow rate due to increased turbulent mixing and diffusion of TAN through the biofilm, however, the percent TAN removal decreased due to decreased hydraulic retention time with increased flow rate.

The kinetic experiments with substrate concentrations at 1, 2, 3, and 4 mg/L in SBSBRs and the modeling of the TAN removal rate fitting Monod model showed that the TAN removal rate followed first order Monod kinetics. This is particularly important in practical sense as the bioreactors' nitrification capacity increases with the increased TAN load into the system that happens with the increasing feed, biomass and subsequent organic loading into a culture system. The experimental and predicted TAN removal rates showed a good fit in the regression model.

The impact of short term organic carbon load into the system showed that the per cent TAN removal rate was decreasing with increased organic load from C/N ratio

0 to 4. However, this decrease was not statistically significant as compared with long term exposure of the biofilters to organic loading in the studies conducted elsewhere.

The kinetic experiments with PBBRs were limited due to the large volumes of feed solution required where as that of SBSBRs were limited due to the difficulty in circulating feed solutions continuously through the system as the reactors operate under immersion into the feed solution.

Validation of the nitrifying bioreactors in recirculating aquaculture systems

In saltwater systems RAS plays an important role in the production of healthy and properly sized fingerlings. Although great efforts have been made on the investigation of nitrifying biofilters for aquaculture applications, most the research have been focusing on performance of an individual component under specific operating conditions using average ammonia removal rate to describe the biofilter nitrification performance. The maintenance of adults and brooders of *P. monodon* in experimental systems without water exchange even at laboratory level was an extremely difficult task as slight alternations in water quality used to lead to mortality. Integration of SBSBRs to the holding system showed that water quality could be maintained to the acceptable level in the rearing systems with out water exchange. Based on these results it has been inferred that SBSBRs can be used in live transportation of spawners and adults from the point of collection to the hatchery and for their quarantine till shifted to the maturation or breeding tank.

Macrobrachium rosenbergii is an inhabitant of fresh water, however, its larval stages are completed in saline waters with salinity 13-15 ppt and therefore, in the larval rearing systems bioreactors operating in this salinity regime are essential. In the PBBR integrated larval rearing systems, ammonia oxidation was established within a day and it took eight days for nitrite oxidation whereas in the control 14 days were required for the initiation of nitrification. The delay in establishing active nitrite oxidation was attributed to the lower multiplication rate of nitrite oxidizers compared to that of ammonia oxidizers. TAN and TNN could be

maintained below 0.25 mg/L, below the maximum set for oligotrophic category and marine larval systems demanded TAN and TNN levels below 1.0 mg/L. In the present field evaluatiion carried out at higher stocking densities (200/L) at a salinty of 15ppt, there was significantly higher percent survival of larvae in the system integrated with the bioreactors when compared to the control. This shall enable hatchery systems to operate as closed recirculating systems.

The integration of packed bed bioreactor for nitrification of hatchery spent water with high percentage removal of ammonia (78%), and nitrite (79%) by 4th day strengthens the possibility of reuse of water with limited discharge and reduced intake paving the way for bio-security. Upon integrating the PBBRs into the maturation system a remarkable reduction in the total ammonia concentration was observed within a week.

The laboratory level recirculating aquaculture system developed integrating PBBRs and indigenous probiotics proved to be successful. In trial 1 the average VTR for the largest feed rate of 150 g was 0.14±0.0083 kgTAN/m^3/day where as in trial 2, the average VTR during the highest feeding rate of 160 g was 0.15±0.0045 kgTAN/m^3/day and these values are comparable to the recommended the values. The maximum volumetric biomass capacity of the system was 68 g/m3 in trial 1 and 75 g/m3 in trial 2. This validation has proved that effective usage of bioreactors and probiotics can maintain a suitable water quality for the culturing of *Penaeus monodon*.

Scope for future research

1. The molecular characterization of the consortia showed the presence of novel organisms in the consortia. This is particularly important as new processes and players are getting recognized in the global nitrogen cycle (Francis et al., 2006). These unique consortia with sustained nitrification capacity under different operating conditions need to be screened extensively with a polyphasic approach.

2. The kinetic studies need to be carried out at extended spatiotemporal scales under different operating conditions. Though simple modeling approaches are enough in the practical sense, considering the long term operation of biofilters under field conditions the kinetic parameters should be estimated following dynamic simulation modeling of the biofilms. This is important as the biofilm itself is defined by the rapidly reproducing heterotrophs while the filters' performance is usually determined by a much more sensitive subpopulation of chemoautrophs, the nitrifiers (Zhang and Bishop, 1994). Studies directed to understand the formation, growth and sloughing off of biofilm from the bioreactors is required for understanding and solving the problems associated with long term operation.

3. To make the recirculation process completely organic, more research is required by supplying organically produced feed to the animal reared in these systems. The Stringed Bed Suspended Bioreactors and Packed Bed Bioreactors developed can form integral parts of Organic Shrimp and Prawn Seed Production system as they are the systems specially designed for tropics using indigenous nitrifying bacterial consortia. Currently organic standards are mainly oriented towards temperate species and it is required to develop organic systems for tropical regions

4. Based on the experimental results a novel Recirculation System for Organic Shrimp and Prawn Seed Production could be designed, principally by integrating the reactors at three locations in a larval production system such as, 1. PBBRs in the reservoir system. 2. PBBRs at brood stock maturation system and 3. SBSBRs at the larval rearing tanks. The probiotic Detrodigest can be used for the management of detritus in the systems. This organism, in association with the heterotrophic bacteria in the nitrifying bacterial consortia, rapidly digests the detritus into monomeric substances and helps attain reef quality water. For the prevention of entry of *Vibrio* sp. through brood stock and nauplii,

Micrococcus MCCB104 could be used. The gut probiotic (Enterotrophotic) can be supplied in all compartments of the larval rearing system such as algal production system, *Artemia* production unit, larval rearing tank and maturation system so as to prevent colonization of the pathogenic *vibrios* any were in the production facility.

To sustain the shrimp industry in India, the production process has to match with the global standards and in this requirement the importance of organically produced good quality seed need not require any more emphasis. Adoption of the above technology in Indian shrimp/ prawn production process is the first step towards the beginning of organic shrimp farming in India and its further expansion into the global scenario.

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ANNEXURE

MEDIA, REAGENTS AND BUFFERS

Electron Microscopy

Reagents

1. **2.5% Glutaradehyde in artificial seawater**

   Volume-20 ml
   Stock-50%

   1ml 50% glutaraldehyde+ 19 ml artificial S W

2. **2% OsO_4**

   Volume- 10 ml
   Stock-4%

   5ml 4% artificial seawater + 5 ml artificial seawater

3. **0.1M Sodium Cacodylate buffer (pH 7.2)**

   Volume- 200ml
   Sodium Cacodylate-4.28 gm
   Solution B= 0.1ml con.HCl + 6.03 DW

   Prepare solution B. Dissolve 4.28 gm of sodium cacodylate in 50 ml distilled water add 4.2 ml of solution B and make to the volume 200ml and check the pH.

4. **Paraformaldehyde-glutaraldehyde-ruthenium red-lysine fixative**

   Volume -20 ml

5. **75mM Lysine**

   1M= 182.65 g/l
   1mM= 182.65 mg/l
   = 0.18265mg/ml
   75mM= 0.18265x75= 13.6988 mg/ml

   In 20 ml= 13.6988x 20= 273.975 mg=0.273975 g

6. **0.080% Ruthenium red**

   0.080gm in 100ml
   In 20 ml= 0.016gm

7. 2%(w/v) Paraformaldehyde

2 gm in 100ml
In 20 ml= 0.4 gm

8. 2.5% Glutaraldehyde

Stock 50%
So 1ml to the 20 ml

DNA extraction

Reagents

Saline EDTA
Lysozyme –Prepare fresh (100mg/ml)
25% SDS
2% proteinase K
Phenol-chloroform
Sodium acetate-3m
Ethanol -100% (ice cold)
Ethanol-70% (ice cold)
Sterile MQ
Rna ase -10mg/ml

9. Saline EDTA (150mM NaCl, 0.1M EDTA (pH 8.0))

EDTA	-	1.862g
Double distilled water	-	40 mL

Adjust the pH using NaOH pellet add NaCl 0.438g; make up the volume to 50mL. Autoclave. Store at 4^0C.

10. 25% SDS (DNA Extraction)

SDS	-	2.5g
Double distilled water	-	10mL

Heat to 68°C and stir with a magnetic stirrer to assist dissolution. Do not autoclave. Store at room temperature.

11. Proteinase K (20mg/mL) (2%)

Proteinase K	-	10mg
Autoclaved distilled water	-	500µL

Dissolve proteinase K in distilled water and store at -20^0C

12. 10mM Tris-Cl (pH8.0)

Tris Base - 0.03028g

D.water - 20mL

Adjust ph to 8.0 using HCl, make up the volume to 25mL, autoclave, and store at 4^0C.

13. Lysozyme (100mg/mL)

Dissolve 10mg lysozyme (for 1 sample) in 100µL of 10mM Tris-Cl (pH 8.0) immediately before use. Make sure pH of Tris solution is 8.0 before dissolving the enzyme otherwise it will not work efficiently if pH is less.

14. 10mM Tris-Cl (pH 8.0)

Tris Base - 0.03028g

D.water - 20mL

Adjust ph to 8.0 using HCl, make up the volume to 25mL, autoclave, and store at 4^0C.

15. 10mM Tris-Cl (pH 7.5)

Tris Base - 0.03028g

D.water - 20mL

Adjust ph to 7.5 using HCl, make up the volume to 25mL, autoclave, and store at 4^0C.

16. RNAase buffer (DNA Extraction)

10mM Tris-Cl (pH7.5) - 10 µL

15mM NaCL (0.8766mg/mL) - 30µL

D. water - 960µL

Autoclave, store at 4^0C

17. RNAase (10mg/mL)

RNAase - 10mg

RNAase buffer, autoclaved - 1mL

Dissolve, heat at 100^0C for 15 mts in boiling water bath, allow to cool at room temperature, store at 4^0C.

18. 3M Sodium Acetate (pH5.2)

Sodium acetate - 12.3g

Double distilled water - 20mL

Adjust the pH to 5.2 using glacial acetic acid, make up the final volume to 50mL , autoclave. Store at 4^0C.

19. Chloroform: Isoamyl alcohol (24:1,v/v)

Store in a light tight bottle.

20. Phenol: Chloroform: Isoamyl alcohol (25:24:1,v/v)

Mix equal volumes of equilibrated phenol and chloroform isoamyl alcohol. Store under 0.1M Tris HCl in a light tight bottle at 4^0C

21. 0.5M Tris- Cl (pH 8.0)

Tris base - 12.112g

Double distilled water - 160ml

Adjust the pH to 8.0 using HCl and make up the volume to 200mL. Autoclave, store at 4^0C.

22. 0.1M Tris- HCl (pH 8.0)

0.5M Tris HCl (pH 8.0) - 50mL

Make up the volume to 250 mL of double distilled water, autoclave and store at 4^0C.

24. Saturation of Phenol with Tris- Cl (pH8.0) (DNA Extraction)

Water saturated phenol - 50mL

a) If Phenol is transparent, add 0.1% 8-hydroxy- quinoline to 50mL of water saturated phenol
b) Cover flask containing phenol with aluminium foil to avoid light reaction.
c) Add 50mL 0.5M Tris- HCl.
d) Stir the solution using magnetic stirrer for 15 minutes
e) Keep the solution for 30 minutes to allow the phenol to settle.
f) Decant the supernatant.
g) Add 50 mL of 0.1m Tris Cl.

h) Repeat the step 4,5,6

i) Repeat step 8 until pH of the phenolic phase is approximately 7.8 (as measured with pH paper)

j) Add 50mL of 0.1M Tris HCl to phenol and store at 4^0C in a light tight bottle.

Phenol is extremely toxic, highly corrossive and cause severe burns!!!!!!!!!!Wear gloves when handling phenol.

PCR cloning Reagents

25. IPTG working stock

Use sterile MCTs and Distilled water or MilliQ.

Dissolve 0.024g IPTG in 1ml of Sterilized distilled water or MilliQ to obtain 0.1M stock solution. Store the stock in -20°C.

26. X-gal working stock

Use sterile MCT wrapped in Aluminium foil. The X-gal solution is light sensitive.

Dissolve 50mg of X-gal in 1ml of N, N'-dimethyl Formamide to obtain 50mg/mL concentration. Store in -20°C.

X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactoside).

27. Ampicillin working stock

Use sterile MCTs and sterile Distilled water or MilliQ.

Dissolve 100mg in 1ml of sterile distilled water to obtain 100mg/ml concentration. Store in -20°C.

28. SOC Medium

2.0g - Bacto-Tryptone
0.5g - Bacto- yeast extract
1ml - 1M NaCl
0.25ml- 1M KCl
1ml - 2M Mg^{2+} stock
1ml - 2M glucose

Add Bacto tryptone, Bacto yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring to 100ml with sterile, distilled water. The final pH should be 7.0.

29. 2M Mg^{2+} stock

20.33g - $MgCl_2.6H2O$
24.65g - $MgSO_4.7H_2O$

Add distilled water to 100mL. Dissolve completely. Filter sterilize. Store at 4°C.

30. 2M Glucose stock

Glucose- 36g

Add distilled water to 100ml. Dissolve completely. Filter sterilize. Store at 4°C

31. 1M NaCl stock

NaCl- 5.8g

Add distilled water to 100ml. Dissolve completely. Sterilize at 121°C for 15 min. Store at Room temperature.

32. 1M KCl stock

KCl – 7.5g

Add distilled water to 100ml. Dissolve completely. Filter sterilize. Store at 4°C

FISH Reagents

Fixation of cells

33. PBS (3X); 390mM Nacl in 30 mM phosphate buffer (pH 7.2)

Dissolve 0.49 g KH_2PO_4 in 80 ml, add 2.3 g NaCl and adjust pH 7.2 Adjust the volume to 100ml

34. PBS (1X); 130mM NaCl in 10 mM phosphate buffer (pH 7.2)

Take 33ml of PBS (3x) and adjust the volume to 100 ml with distilled water.

35. 4% paraformaldehyde in PBS

a) Heat 6.5 ml milliQ to 60 C (Normally it is sufficient to warm it by hot running tap water)
b) Add 0.4 g Paraformaldehyde
c) Add one drop of 1M NaOH and shake vigorously until the solution has nearly clarified (1-2 min)

d) Remove the solution from the heat source and add 3.3 ml 3X PBS

e) Adjust the pH to 7.2 with HCl (one drop 1M HCl)

f) Filter the solution through 0.2µm membrane disc filter

g) Keep the solution on ice until used or store at -20° C

(Caution 1: Paraformaldehyde is very toxic, use gloves, Caution 2: Use only freshly prepared fixative (<24h old), or deep frozen out of the -20° C)

36. 98% Ethanol at -20

37. 50%, 80% and 98% ethanol

38. MilliQ at 4

39. 1M NaOH

Dissolve 4 g of NaOH in 80 ml distilled water; adjust the volume to 100 ml 1M HCl

Hybridization

40. 10% KOH in 95%ethanol

Dissolve 10 g KOH in 95% ethanol.

41. 0.1% gelatin solution in 0.01% Chromium potassium sulfate dodecahydrate

Dissolve 0.1 g gelatin and 0.01g chromium potassium sulfate dodecahydrate in 100 ml MilliQ.

42. 1M Tris/HCl (pH 8.0)

Dissolve 12.1 g Tris base in and adjust the pH to 8 with HCl, adjust the volume to 100 ml.

43. Formamide

(Use formamide only in the fume and wear gloves!!)

44. 0.5 M Na_2EDTA (pH, 8)

Dissolve 18.1g Na_2EDTA in 80 ml , adjust to pH 8.0 and adjust volume to 100ml

45. 10%(v/v) SDS

Dissolve 2 g of sodiumdodecylsulfate in 20 ml of milli Q.

Preparation of hybridization buffer for insitu hybridization at 46°C

Pipet into a 2ml eppendorf tube
5M NaCl -360 µl
1M Tris/HCl (pH 8.0)- 40µl
Add formamide and milliQ according to the following table:

% (v/v) Formamide	Formamide (µl)	MilliQ (µl)
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400
65	1300	300

10% (w/v) SDS- 4µl

Preparation of washing buffer for in situ hybridization at 46°C (Washing at 48° C, 20 min)

Pipet into a 50 ml falcon tube and mix:

Tris/HCl (pH 8.0)- 1 ml
Add 5 M NaCl and 0.5M EDTA (pH 8.0) according to the following table

% (v/v) Formamide in hybridization buffer	NaCl (mM)	5M NaCl (µl)	0.5 M EDTA(µl)
0	0.900	9000	
5	0.636	6300	
10	0.450	4500	
15	.0318	3180	
20	0.225	2250	500
25	0.159	1590	500
30	0.112	1120	500
35	0.080	800	500
40	0.056	560	500
45	0.040	400	500
50	0.028	280	500
55	0.020	200	500
60	0.008	80	500
70	0.000	0	350

Fill the falcon tube upto 50 ml with milliQ
Add 50 µl of 10% (w/v) SDS
Preheat the washing buffer at 48° C prior to use

LIST OF PUBLICATIONS

Publications out of this thesis

Rejish Kumar VJ, Poulose G, Sudheer NS, Singh ISB. 2006. Organic recirculating aquaculture-A lab scale demonstration. 7th Asia Pacific Marine Biotechnology Conference 2nd-5th November 2006, Kochi, India. p.119

Kumar VJR, Achuthan C, Manju NJ, Philip, R, Singh ISB. A stringed bed suspended bioreactor (SBSBR) for in situ nitrification in penaeid and non-penaeid hatchery systems. Aquaculture International (Submitted).

Kumar VJR, Achuthan C, Manju NJ, Philip, R, Singh ISB. A packed bed bioreactor (PBBR) for nitrification in *Macrobrachium rosenbergii* larval rearing systems. World Journal of Aquaculture. (Submitted).

Kumar VJR, Achuthan C, Manju NJ, Philip, R, Singh ISB. Mass production of nitrifying bacterial consortia for immobilization in bioreactors for penaeid and non-penaeid hatchery systems. Journal of Industrial Microbiology and Biotechnology (Manuscript).

Molecular characterization of nitrifying bacterial consortia used for activation of nitrifying bioreactors in aquaculture systems (Manuscript under preparation)

Publications co-authored

Achuthan C, **Kumar VJR**, Manju NJ, Philip R, Singh ISB (2006) Development of nitrifying bacterial consortia for immobilizing in nitrifying bioreactors designed for penaeid and non-penaeid larval rearing systems in the tropics. Indian Journal of Marine Sciences 35(3) 240-248.

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