Development of nitrifying and photosynthetic sulfur bacteria based bioaugmentation systems for the bioremediation of ammonia and hydrogen sulphide in shrimp culture

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By

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October 2007

CERTIFICATE

This is to certify that the research work presented in this thesis entitled ***"Development of nitrifying and photosynthetic sulfur bacteria based bioaugmentation systems for the bioremediation of ammonia and hydrogen sulphide in shrimp culture"*** is based on the original work done by Ms. Manju N. J. (Reg. No. 2431) under my supervision at National Centre for Aquatic Animal Health, School of Environmental Studies, Cochin University of Science and Technology, Cochin 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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DECLARATION

The research work presented in this thesis entitled ***"Development of nitrifying and photosynthetic sulfur bacteria based bioaugmentation systems for the bioremediation of ammonia and hydrogen sulphide in shrimp culture"*** submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, is a bonafide record of the research work done by me under the supervision of Dr. I. S. Bright Singh, Professor in microbiology, Director, School of Environmental Studies, Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin 682016. 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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Dedication.....

"Lack of analytical, gender-specific information has worked to inhibit development opportunities for women in fisheries sector and this has resulted in economic planners not viewing women as stakeholders. Part of the reason for this is that much of the work done by women is not remunerated or is poorly remunerated and, therefore, little valued in financial terms."

Dr. Merryl J. Williams,
Former Director General,
World Fish Centre, Penang, Malaysia

On, why women's contributions are hardly recognized despite strong resourseſulness and capacity in fisheries sector.
I dedicate my humble work to those women folks.....

CONTENTS

Abbreviations

AHL	Acyl homoserine lactones.
AMNS	Acclimated marine nitrifying sludge.
AMO	Ammonia monooxygenase.
AMONPCU	Ammonia oxidizing consortia for non-penaeid culture systems.
AMOPCU	Ammonia oxidizing consortia for penaeid culture systems.
ANOVA	Analysis of variance.
AOA	Ammonia oxidizing archaea.
AOB	Ammonia oxidizing bacteria.
ATP	Adinosine triphosphate.
bchl	Bacteriochlorophyll.
BDL	Below Detectable Limit
BET	Brunauer-Emmett-Teller.
bp	Base pair
BOD	Biochemical oxygen demand.
CLSM	Confocal laser scanning microscopy.
COD	Chemical oxygen demand.
Cv	Coefficient of variance.
DAPI	4,6-diamidino-2-phenylindole.
DGGE	Denaturing gradient gel electrophoresis.
DIN	Dissolved inorganic nitrogen.
DNA	Deoxyribonucleic acid.
dNTP	Deoxy nucleotide triphosphate.
DO	Dissolved oxygen.
EDTA	Ethylene diamino tetra acetic acid.
EPS	Extracellular polymeric substances.
FCR	Food conversion ratio.
Fig.	Figure.
FISH	Fluorescent in situ hybridization.
GSB	Green sulphur bacteria.
HAO	Hydroxylamine oxidoreductase.
MPN	Most probable number.
nar	Nitrate reductase.
NBC	Nitrifying bacterial consortia.
NBP	Nitrifying bioaugmentation product.
NCAAH	National centre for aquatic animal health.
NIONPCU	Nitrite oxidizing consortia for non-penaeid culture systems.
NIOPCU	Nitrite oxidizing consortia for penaeid culture systems.
nir	Nitrite reductase.
NO	Nitric oxide.
NOB	Nitrite oxidizing bacteria.
nor	Nitric oxide reductase.
nos	Nitrous oxide reductase.
OD	Optical density.
OLAND	Oxygen limited aerobic nitrification and denitrification.
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction.

ppm	Parts per million.
ppt	Parts per thousand.
PSB	Purple sulphur bacteria.
PVP	Polyvinyl pyrrolidone.
RAS	Recirculation aquaculture system.
RBC	Rotating biological contactors.
rDNA	Ribosomal deoxynucleic acid.
RNA	Ribonucleic acid.
RT-PCR	Reverse transcriptase- Polymerase chain reaction.
SDS	Sodium dodecyl sulphate.
SEM	Scanning electron microscopy.
TAN	Total ammonia nitrogen.
TEM	Transmission electron microscopy.
TNN	Total nitrite nitrogen.
UV	Ultra violet.

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Chapter 1
General introduction

1.0. General Introduction.

1.1. Aquaculture – an overview

FAO has defined aquaculture as "the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc." (FAO, 1998). FAO has collectively termed the land-based and water-based brackish and marine aquaculture practices as 'coastal aquaculture' (FAO, 1992). Aquaculture has also been defined as 'the rearing of aquatic organisms under controlled or semi-controlled conditions'' (Landan, 1992). More than 220 species of finfish and shellfish are farmed. In extensive aquaculture, interventions are limited to exclusion of predators and control of competitors, and it turns to semi-intensive when some strategy to enhance food supply is adopted. Intensive aquaculture practice provides provision for all nutritional requirements, which demands greater use of management of inputs, waste generation and disease (Naylor *et al.* 2000). Aquaculture is now an important economic activity world wide. As per the UN, FAO studies, half of the world's seafood demand will be met by aquaculture in 2020, as wild capture fisheries are over exploited and are in decline.

Aquaculture has become an important economic activity in many tropical countries and Asia contributes 80% of the world's production (Csavas, 1994). Over the past two decades the industry has gone through major changes from a small scale operation to large scale commercial ventures. Production from aquaculture often exceeds landings from marine capture fisheries in many areas (NACA/ FAO, 2001). Annual average rate of output from aquaculture, excluding aquatic plants; was at a rate of 9.1% while marine capture fisheries grew at a rate of 1.2% only. This faster growth rate indeed out competes any other animal food producing systems such as capture fisheries and farmed meat (FAO, 2003). The trend is to continue as the population keeps on growing with a per capita increase in the consumption of seafood as other protein consumption decreases. This pressing need drives the industry towards the adoption of intensive culture practices. While doing so water quality and quantity constraints, availability and cost of land, regulations on water discharge and the concern of environmental impacts do highly influence the kind of technology to be adopted for upgradation (Gutierrez-Wing & Malone, 2006). Recirculating technology is a promising solution amid such constraints; it reduces water demands and discharges by reconditioning of water (Goldburg *et al.* 2001). A

recirculation aquaculture system (RAS) maintains better food conversions and reduces waste generation from left over feed (Lorsordo *et al.* 1998). Similar is the case with zero water exchange or minimal water exchange systems where all above requirements are satisfied (Burford *et al.* 2003; Balasubramanian *et al.* 2004).

1.1.1. Shrimp aquaculture and its impact on environment

Shrimp culture is widespread throughout the tropical world. Commercial shrimp farming has been accelerated due to huge demand for fin and shellfish in the international market, and to meet the global demand for shrimp; 85% of the production is based on intensive cultivation practice. This ever growing demand and success registered by countries like Thailand made other Asian countries including India to gear up their own programmes to develop shrimp farming industry as a major commercial venture. The very nature of intensified shrimp culture is its dependence on a lot of inputs (Deb, 1998). Ultra-high stocking densities and feed loading are the typical characteristics of intensive culture practices (Shang *et al.* 1998). The uncertainty in relation to climatic changes, huge capital investment and ever increasing dependence to surrounding environment make the aquaculturists follow the principle of adopting short-cut ways to profit making, ultimately giving rise to numerous impacts (Funge-Smith & Briggs, 1998). In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses (Balc'azar *et al.* 2006). Chemicals and other substances are extensively used as additives for improving water quality, disease control and against other biological problems like, algal bloom, aquatic plant infestations, controlling vectors and proliferation of weed species (Boyd, 1995a). All these additives, fertilizers and management chemicals ultimately find its way out to the surrounding environment at the end of each production cycle. Problems due to such discharge have serious implications on the fragile coastal stretches. Due to these situations the rapid and unscientific development of the shrimp culture in the tropical countries led to adverse environmental impacts (Shan & Obbard, 2001). Consequently serious production losses occurred in almost all shrimp producing countries around the world including India.

The problem of nutrient loading in sea/ creek due to the discharge of shrimp culture wastes has been studied extensively in many countries. Shrimp culture wastewater comprises both living and dead plankton, bacteria, feed waste, faecal matter, and other excretory products of shrimps. Though all these nutrients and organic wastes are

biodegradable, the soluble nutrients such as nitrogen, ammonia and phosphorus, beyond a reasonable limit, can result in nutrient enrichment in the open waters. This in turn affects the quality of source water and increases the risk involved in shrimp culture. Typically shrimp farming enjoys an initial period of success and good production followed by gradual decrease in yield over successive crops (Funge-Smith & Briggs, 1998). Deterioration of the source water quality is cited as one of the reasons. Depending on a plethora of factors, decreased yields are manifested as reduced growth, higher FCR, disease outbreak and consequent emergency harvest. Under such conditions shrimp farmers are forced to use several chemicals and biological products to attain sustainability and to increase yield. This scenario will eventually lead to the deterioration of the system. Adverse impact of uncontrolled and unplanned shrimp farming and its direct implication on the coastal ecosystem of Bangladesh has been well depicted by Deb (1998).

The requirement of prevention and control of diseases under such deteriorating conditions have led to substantial increase in the use of veterinary medicines in the recent decades. Along them, the utility of antimicrobial agents as a preventive measure has been questioned, as there is extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria and its transfer to human pathogens (Moriarty, 1999). Antibiotic resistance mechanisms can arise either due to chromosomal mutation or acquisition of plasmids. Chromosomal mutations cannot be transferred to other bacteria but plasmids can transfer resistance rapidly. Several bacterial pathogens can develop plasmid-mediated resistance, and plasmids carrying genes for resistance to antibiotics have been found in marine *Vibrio* species and they could be laterally exchanged.

In order to reduce the negative impacts of aquaculture, several countries have already adopted policies to bring down the impairment of aquatic environments through environment friendly technological interventions. Conference on Aquaculture in the third millennium, on February 2000 issued the *Bangkok declaration and strategy* (BDS), which recognized that policies and regulations should promote practical and economically viable farming and management practices that are environmentally acceptable and socially responsible. The challenge for sustainable industrial growth is to improve production performance while, at the same time, to minimize the environmental impacts (Martinez-Cordero & Leung, 2004).

Table 1.1: Standard for shrimp aquaculture wastewater

SLNo.	Parameters	Guidelines issued by the Ministry of Agriculture		General standards for discharge of environmental pollutants[87]
		Coastal marine waters	Creek	Marine Coastal areas
1.	pH	6.0-8.5	6.0-8.5	5.5 – 9.0
2.	Suspended solids (mg/l)	100	100	100
3.	Dissolved oxygen (mg/l)	not less than 3	Not less than 3	-
4.	Free Ammonia(as NH3-N) (mg/l)	1.0	0.5	5
5.	Biochemical oxygen Demand- BOD(5 days @ 20 c)	50	20	100
6.	Chemical Oxygen Demand-COD (mg/l)	100	75	250
7.	Dissoved Phospate (as P) (mg/l max)	0.4	0.2	-
8.	Total Nitrogen (as N) (mg/l)	2.0	2.0	-

Source: Guidelines for Sustainable Development and Mangement of Brackishwater Aquaculture. Ministry of Agriculture, GOI.

1.1.2. Sustainable aquaculture

Shrimp aquaculture would not sustain the growth, as a major food source for the ever increasing population, unless it adopts more environment friendly approaches. Disease management, productivity and environment protection are three key points for successful shrimp culture with sustainability. Accumulation of detritus in the pond bottom either uniformly or in pockets, generation of anoxic zones, production of hydrogen sulfide and building up of unionized ammonia are the major events which take place in shrimp grow out systems. Recent emergence of potential pathogens like white spot virus turned out water exchange risky as at any moment pathogen can enter and spell havoc. Moreover, minimal water exchange is recommended to minimize the impact of aquaculture in the surrounding environment. To reduce the cost of shrimp production, in the place of frequent application of fertilizers, mineralization and regeneration of nutrients and application of the required and essential nutrients have become the rule of thumb in attaining sustainable primary and secondary productivity.

This implies that to protect shrimp culture from potential pathogens such as white spot syndrome virus and to save the coastal zone from the impact of shrimp culture the option before is to resort to a closed culture system with zero water exchange. In such a situation the culture operation must be under comprehensive bioremediation package having these discrete components such as detritus management, nitrification and hydrogen sulphide assimilation/ oxidation, appropriately integrated.

1.1.3. Nitrogen flux and coastal ecosystems

The process of nitrification is important in aquatic environments, because it links nitrogen mineralization, which results in the formation of ammonia from organic materials, to denitrification, which results in the loss of nitrogen from the system in the form of gaseous dinitrogen. This process is especially important in coastal aquatic systems where anthropogenic inputs of nitrogen can result in heavy nutrient loading, and denitrification can account for the loss of up to 50% of the inorganic nitrogen input. Disruption of nitrification and/or denitrification can lead to eutrophication and system degradation (Mullan and Ward, 2005).

More specifically adverse environmental impacts associated with high ammonia-nitrogen load in aquatic bodies, includes promotion of eutrophication, toxicity to aquatic organisms and depletion of dissolved oxygen due to bacterial oxidation of ammonia to nitrite and nitrate (Klees and Silverstein, 1992). Coastal regions often receive high loads of nitrogen due to various anthropogenic activities. This large input of nitrogen invariably leads to eutrophication and such addition has a profound effect in estuaries and lagoons with limited water exchange (Herbert, 1999). Such nitrogen input promotes fast growing pelagic micro algae and phytoplankton which in turn effects benthic primary production due to poor light penetration. This shift from benthic to pelagic primary production sets in with large diurnal variations in dissolved oxygen. This also induces high oxygen consumption in the sediments due to the deposition of highly degradable biomass (Herbert, 1999). Microbial communities in shallow sediments of marine and brackish water ecosystems play an important role in the degradation of complex organic compounds and regeneration of nutrients to sustain primary production in the overlying water column. Significance of these intricate processes has been exemplified by the biogeochemical cycling of nitrogen involving specialized groups of microorganisms.

Nitrogen components play a key role in aquaculture water quality management, as they assume a dual role as nutrients and toxicants in aquaculture environment (Lorenzen *et al.* 1997). Traditional and semi intensive aquaculture systems heavily depend on primary production and often make deliberate inputs of nitrogen in the form of fertilizers to enhance primary production. In intensive culture systems, production entirely depends on input of organic nitrogen as commercial feeds and management aims at reducing the concentration of toxic nitrogen compounds inevitably added to the water by excretion and feed loss (Lorenzen *et al.* 1997). Mathematical models have been used to elucidate a complete picture on nitrogen dynamics in intensive pond cultures (Lorenzen *et al.* 1997).

This model, calibrated for two shrimp farms in Thailand describes the input of ammonia, its assimilation by phytoplankton or nitrification and the loss of nitrogen by sedimentation, volatilization and discharge. Assimilation by phytoplankton with subsequent sedimentation or discharge is the important process of ammonia removal. Nitrification and volatilization sets in when the ammonia input exceeds the algal assimilation capacity. Nitrification is an important process of nitrogen cycle in aquatic ecosystems, nitrifying bacteria and phytoplanktons have a unique correlation in their use of ammonia and nitrite (Feliatra *et al.* 2004). In environments with high nutrient inputs, mineralization of organic matter, overfeeding and excretion increases the ammonia concentration which is harmful for fish and shrimp (Goldman *et al.* 1985). High nitrogen outputs may trigger eutrophication, as nitrogen is a major limiting nutrient (Dugdale, 1967). Nitrification when coupled with denitrification, alleviate the effects of eutrophication through removal of nitrogen to the atmosphere as nitrous oxide and dinitrogen gas (Blackburn & Blackburn, 1992). In situations of chronic pollution, nitrification and subsequently denitrification are often suppressed, magnifying the environmental impact of the aquaculture industry (Kaspar *et al.* 1988).

The process of biological nitrogen removal involves aerobic nitrification and anaerobic denitrification processes. But these processes in application level require contrasting hydraulic retention time and dissolved oxygen concentration, and such extreme requirements make the efficiency improvement an uphill task (Nishio *et al.* 1998). Autotrophic nitrifiers may not tolerate induced redox changes for simultaneous nitrification and denitrification process, this may cut short the performance of such processes.

1.1.4. Ammonia/nitrite toxicity

Water quality and economics of shrimp culture are closely related to efficiency of nitrogen assimilation by cultured species. Dissolved inorganic nitrogen (DIN) is second only to dissolved oxygen (DO) as a factor that most likely to limit feeding rate in culture ponds. Intensive aquaculture relies on high protein content feeds (Lovel, 1989) that are rich in nitrogen (5-6.5%N) and often feed application in these systems reaches 60g/m^3/day (Creswell, 1993). Most of the applied nitrogen (approximately 70%) remains in the pond water as uneaten feed and fish excreta (Boyd, 1985; Gross *et al.* 2000). About 11 to 36% (average 25%) of nitrogen added as feed or other nutrients is recovered by the cultured species (Hargreaves, 1998). Around 90% of assimilated nitrogen by marine shrimp is

excreted as TAN (total ammonia nitrogen) and urea (Ebeling *et al.* 2006). Bacteria transform the nitrogen in uneaten feed to ammonia and fish excrete ammonia through their gills. Ammonia is toxic even at low concentration. Recommended safe ammonia concentration in aquaculture is lower than 0.02- 0.3 mg/L (Boyd and Tucker, 1998) and for nitrite it is lower than 1mg/L.

In aqueous solution both unionized ammonia (NH_3) and ionized ammonia (NH_4^+) exist in equilibrium. The proportion of NH_3 to NH_4^+ in water increases with increase in water temperature and pH and with decrease in salinity (Trussel, 1972; Whitfield, 1978). As pH turns less acidic, concentration of toxic unionized ammonia increases logarithmically. The sum of un-ionized and ionized ammonia (NH_3 + NH_4^+) is usually referred to as total ammonia-nitrogen (TAN). Sometimes the term 'ammonia' is used to refer the total ammonia-nitrogen (Ebeling *et al.* 2006). NH_3 is lipophilic and easily diffusible across cell membranes. By contrast, NH_4^+ is lipophobic and penetrates membranes less readily (Kormanik and Cameron, 1981) and is toxic, at low pH levels (Allen *et al.* 1990). Elevated environmental ammonia-nitrogen has been reported to affect growth and moulting (Chen and Kon, 1992), oxygen consumption, ammonia nitrogen excretion (Chen and Lin 1992) and Na^+, K^+-ATPase activities in penaeids (Chen and Nan, 1992). Accumulation of nitrite in water has been reported to cause reduction of hemolymph and oxy- hemocyanin in *Penaeus monodon* (Chen and Cheng, 1995; Cheng and Chen, 1999). Elevated nitrite levels cause brown blood disease or methaemoglobinemia. Being a weak base, NH_3 binds with a proton and raises the intracellular pH upon entering the cell. This intracellular alkalinization would affect the normal biochemical reactions, as enzymes are generally pH-dependent. For fish both ammonia and nitrite are toxic. Ammonia toxicity is thought to occur from osmoregulatory imbalance causing renal failure and gill epithelial damage resulting in suffocation, decreased excretion of endogenous ammonia and general neurological and cytological failure (Meade 1985).

1.1.5. Amelioration of ammonia/nitrite toxicity

One of the common practices for ammonia and nitrite removal is a large water exchange. High water exchange leads to large and costly water consumption, disease outbreaks, release of saline effluents containing all those management chemicals and additives. Selective ion exchange columns are used to mitigate ammonia toxicity in aquaculture, naturally occurring zeolite, at pH 6.5 has a high selectivity for ammonium.

However this method was not efficient to control ammonia concentration well below the safe limit and they cannot be used for the removal of nitrite and nitrates. It is also obvious that the ion exchange media has a certain capacity, and it requires frequent recharge. Because it is an ion exchange system and salt water has very high ion concentrations, ion exchange will not work in salt or most brackish water systems. It works well in fresh water systems, but there is concern about the cost as ion exchange media is quite expensive. There can be a plethora of variables that affect the efficiency or removal of specific ions by ion exchange media.

The most widely used method for alleviating ammonia problem in aquaculture is biological nitrification using biofilters or biofilm reactors. A closed recirculating aquaculture system with biological nitrification process offers an opportunity. The rate at which the conversion of ammonia to nitrate takes place is dependent on nitrogen concentration, dissolved oxygen, organic matter, temperature, and other variables. Although the nitrification process has been intensively studied during the past several decades, there are many treatment plants that fail to establish stable nitrification (Mertoglu *et al.* 2005). This is mainly due to low maximum growth rates of nitrifiers and their high sensitivity to toxic shocks and sudden pH and temperature changes during the operational period.

Commercial microbial amendments claiming to be nitrifying bacteria are marketed as liquid microbial consortia, for reducing ammonia levels in hatcheries and aquaculture ponds. Some of the products have listed autotrophic nitrifiers as an active ingredient along with other heterotrophic bacteria like *Bacillus, Pseudomonas* etc which can be administered as a pond additive to enhance water quality. There are products which claim to contain nitrifiers, need brewing before application. Several such products are not backed with any scientific study and claims are not validated by research.

1.1.6. Recirculatory aquaculture system

Recirculating aquaculture systems (RAS) are biologically intensive with high stocking densities. Privilages of a recirculatory aquaculture system are its greatly reduced land and water requirements; high degree of environmental control, optimal growth rates, through out the year and the feasibility of locating in proximity to markets (Masser *et al.* 1999). Performance deterioration due to poor design, sub par management and flawed economics are the major bottlenecks. Recirculating systems are mechanically sophisticated and biologically complex (Masser *et al.* 1999). Management of such

demanding systems requires some level of education and expertise. Poorly managed systems experience, component failures, poor water quality, off flavour, stress and disease. The main disadvantage of recirculation is the initial capital cost for installation of pumps, filters, sumps and reliable electrical supply.

1.2. Nitrification

Nitrification is an important part of the biological nitrogen cycle. The nitrification process is defined as the biological transformation of reduced forms of nitrogen to nitrate. It is a microbial process by which reduced nitrogen compounds are sequentially oxidized to nitrite and nitrate. It is accomplished by two groups of autotrophic nitrifying bacteria that can build organic molecules using energy obtained from inorganic sources like ammonia or nitrite. The most important groups of organisms, involved in this process, are the lithoautrophic ammonia-oxidizing bacteria and the litho autotrophic nitrite-oxidizing bacteria (Koops and Pommerening-Röser, 2001). Besides, many other heterotrophic, autotrophic bacteria and archaea actively contribute to biological nitrogen removal process.

Chemolithotrophic nitrification is a rate limited two step process, which involves conversion of ammonia to nitrite and production of nitrate from nitrite (Kowalchuk and Stephen, 2001). These conversions are carried out by two groups of highly specialized organisms – the ammonia oxidizers and the nitrite oxidizers – collectively called nitrifiers. Members of ammonia oxidizing bacterial group (AOB) have the prefix *Nitroso*, whereas those of nitrite oxidizing bacterial group (NOB) start with *Nitro*. These organisms grow lithotrophically utilizing energy generated by the oxidation of ammonia to nitrite (ammonia oxidizers) or nitrite to nitrate (nitrite oxidizers). Nitrifiers are autotrophic bacteria, fixing CO_2 as the main carbon source via the Calvin cycle. There are no known autotrophic bacteria that can catalyze the production of nitrate from ammonia in a single step (Kowalchuk and Stephen, 2001, Tamegai *et al.* 2007). Such autotrophic bacteria that can oxidize ammonia directly to nitrate have not been described yet, although recently Costa *et al.* (2006) postulated that bacteria that completely oxidize ammonia to nitrate, referred to as complete ammonium oxidation (comammox), exist in the environment.

Oxidation of inorganic nitrogen compounds serve as characteristic energy source for the two distinct groups of chemolithoautotrophic nitrifying organisms and together they catalyze the two step oxidation process. In the first step of nitrification, ammonia-oxidizing bacteria oxidize ammonia to nitrite.

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

Nitrosomonas is the most frequently identified genus associated with this step, although other genera, including *Nitrosococcus*, and *Nitrosospira* can also oxidize ammonia to nitrite. In the second step of the process, nitrite-oxidizing bacteria oxidize nitrite to nitrate.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

Nitrobacter is the most referred genus associated with the second step of nitrification. Other genera including *Nitrospina*, *Nitrococcus*, and *Nitrospira* are also associated with autotrophic oxidation of nitrite (Watson *et al.* 1981). The stoichiometry of nitrification reactions is described by the following Mole-balanced equations (Chandran and Smets, 2000).

$$55NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7NO_2 + 54NO_2^- + 57H_2O + 104H_2CO_3$$

$$400NO_2^- + NH_4^+ + 195O_2 + 4H_2CO_3 + HCO_3^- \rightarrow C_5H_7NO_2 + 3H_2O + 400NO_3^-$$

Chemolithoautotrophic nitrifiers can be found in diverse environmental conditions, they have been isolated from many ecosystems such as fresh water, salt water, sewage systems, soils and on rocks as well as in masonry (Watson *et al.* 1989). Nitrifiers can also be found in extreme habitats at high temperatures (Egorova and Loginova, 1975) and in Antarctic soils (Arrigo *et al.* 1995, Wilson *et al.* 1997). 16S rRNA gene sequence of dominant β-Proteobacterial AOB from the polar oceans has also been observed as a dominant sequence in clone libraries from the Mediterranean Sea (Mullan and Ward, 2005), which recently spurred interest on the diversity and composition of marine AOB assemblages. Though the optimal pH preference is 7.6 to 7.8 and they are frequently detected in environments with low pH values such as acid tea soil (pH 4.0) and forest soils (De Boer and Kowalchuk, 2001, Hart, 2006). Nitrifiers were also detected at high pH values of about 10.0 such as highly alkaline soda lakes (Sorokin *et al.* 2001, Sorokin *et al.* 1998). Under suboptimal conditions, growth might be possible by ureolytic activity, aggregate formation (De Boer *et al.* 1991) or by biofilm formation on the surface of substrate.

Another interesting fact regarding aerobic nitrifiers is that they were found even in anoxic environment (Weber *et al.* 2001). Presence of anaerobic metabolism (Abeliovich and Vonhak, 1992) and reports on the presence of aerobic nitrifiers in anoxic conditions

are in good agreement with recent studies, emphasizing the presence of more versatile metabolism than previously assumed (Schmidt *et al.* 2002). Metabolism of ammonia oxidizing bacteria is surprisingly versatile. Ammonia oxidizers can denitrify with ammonia as electron donor under oxygen limited conditions (Goreau *et al.* 1980 and Kuai and Verstraete, 1998) or with hydrogen or organic compounds under anoxic conditions (Bock *et al.* 1995). The free energy of such a reaction where ammonium serves as electron donor is equivalent to that of the nitrification process (Van Loosdrecht and Jetten, 1998). The conversion of ammonia to nitrite yields little energy due to the high standard redox potentials of the two redox couples NH_2OH/NH_3 (+899 mV) and NO_2^-/NH_2OH (+66 mV). Consequently, ammonia-oxidizers are slow-growing bacteria (Daims, 2001). Anoxic reduction of nitrite (denitrification) by *Nitrosomonas europaea* with pyruvate as electron donor has been observed (Abeliovich and Vonhak, 1992), and *N. eutropha* can reduce nitrite with hydrogen as electron donor at low oxygen pressure (Bock *et al.*, 1995). Pathway by which AOB perform the reduction of nitrite to nitric oxide and nitrous oxide was termed as 'nitrifier denitrification' pathway, and it was recently exemplified in the case of *Nitrosospira* (Shaw *et al.* 2006).

1.2.1. Chemolitho-autotrophic ammonia oxidation

Conversion of ammonia to nitrite proceeding through hydroxylamine by ammonia oxidizing bacteria (AOB) can be depicted by the following equations.

$$2H^+ + NH_3 + 2e^- + O_2 \rightarrow NH_2OH + H_2O \qquad (1)$$

$$NH_2OH + H_2O \rightarrow HONO + 4e^- + 4H^+ \qquad (2)$$

$$2H^+ + {}^1/_2O_2 + 2e^- \rightarrow H_2O \qquad (3)$$

A membrane bound multi-subunit enzyme, ammonia monooxygenase (AMO) mediates the first reaction depicted in equation (1) and hydroxylamine oxidoreductase (HAO), a periplasm associated enzyme catalyzes the 2nd reaction. Only the oxidation of hydroxylamine is exergonic and is therefore regarded as the actual energy source in lithotrophic ammonia oxidation. Two electrons are used to compensate the electron input in the first reaction and the other two electrons are passed to the terminal oxidase via an electron transport chain. The overall reaction can be represented by the equation (4).

1.2.1.1. Lithotrophic ammonia-oxidizing bacteria

AOB are obligate aerobes, but some species may be remarkably tolerant to low oxygen conditions and even anoxic conditions (Kowalchuk and Stephen, 2001). This group of bacteria mediates the first step of nitrification, conversion of ammonia to nitrite. Two phylogenetically distinct groups are defined, one within the γ subclass of the Proteobacteria and other in the β subclass of the Proteobacteria. Genus *Nitrosococcus*, which belongs to the γ Proteobacteria is represented by two well described species, *Nitrosococcus oceani* and *Nitrosococcus halophilus* (Koops *et al.*1990). The β subclass group consist of two clusters, the *Nitrosospira* cluster with closely related members in three genera and the *Nitrosomonas* cluster with at least five distinct lineage of descent (Pommerening-Röser *et al.*1996).

1.2.2. Chemolitho-autotrophic nitrite oxidation

Nitrite-oxidizing bacteria perform the second step of nitrification, the oxidation of nitrite to nitrate. This group of bacteria is phylogenetically more heterogeneous as all of the four described genera belongs to different lines of descent. The membrane bound enzyme nitrite oxidoreductase catalyses the chemolithotrophic oxidation of nitrite to nitrate. This reaction is reversible and the oxygen, incorporated to nitrate is derived from water (equation 5)

$$NO_2^- + H_2O \leftrightarrow NO_3^- + 2H^+ + 2e^- \qquad (5)$$

1.2.2.1. Lithotrophic nitrite-oxidizing bacteria

These bacteria catalyze the second step in nitrification by oxidizing nitrite to nitrate. There are four phylogenetically distinct groups of nitrite-oxidizers. The genus *Nitrobacter* represents a major group, belongs to α subclass of the Proteobacteria. Two marine species, *Nitrococcus mobilis* and *Nitrospina gracilis* (Watson and Waterbury, 1971) were assigned to the γ and the δ subclass of the Proteobacteria, respectively. The two species of the genus *Nitrospira* are members of a distinct phylum close to the δ subclass of the Proteobacteria. Only *Nitrobacter* is able to grow heterotrophically (Burrell *et al.* 1998).

1.2.3. Heterotrophic nitrification

Heterotrophic nitrification is catalyzed by several organisms, comprising fungi as well as heterotrophic bacteria. Under heterotrophic nitrification, both inorganic and organic pathways have been postulated with little knowledge about biochemical reactions involved (Killham, 1986). It is uncertain whether heterotrophic nitrification is via an inorganic pathway involving hydroxylamine and nitrite or via an organic pathway involving oxidation of an amine or amide to a substituted hydroxylamine and with subsequent oxidation to a nitroso- and then to a nitro- compound (Nugroho, 2006). Or it may be a combined inorganic and organic pathway (Killham, 1990).

Inorganic heterotrophic nitrification pathway ($NH_4^+ \rightarrow NH_2OH \rightarrow NOH \rightarrow NO_2^- \rightarrow NO_3^-$)

Organic heterotrophic nitrification pathway ($RNH_2 \rightarrow RNHOH \rightarrow RNO \rightarrow RNO_2 \rightarrow NO_3$)

It is now evident that a variety of heterotrophs oxidize nitrogen compounds (Hirsch *et al.* 1961). These organisms oxidize a number of organic nitrogenous substances apart from ammonium, hydroxylamine and nitrite (Verstraete and Alexander, 1972). Activities of heterotrophic nitrification have been thought to be low relative to the activity of autotrophic nitrifiers (Castignetti and Hollocher, 1984). In recent years heterotrophic nitrification has been targeted as a foolproof solution for simultaneous nitrification denitrification process, as many of the heterotrophic nitrifiers found to denitrify their nitrification products simultaneously under aerobic conditions. Often this attribute was the factor that led to the underestimation of their heterotrophic nitrification activities. Heterotrophic nitrifiers like *Paracoccus denitrificans,* (formerly *Thiosphaera pantotropha*) was used in activated sludge (Kshirsagar *et al.* 1995) and their immobilized forms (Dalsgaard *et al.* 1995) were used to improve nitrogen removal in waste water treatment systems. *P. denitrificans* possess single linked copies of gene encoding AMO and HAO (Crossman *et al.* 1997).

1.2.4. Anaerobic ammonia oxidation (anammox)

A new group of anaerobic nitrite-dependent ammonia oxidizers (anammox) were discovered (Mulder *et al.* 1995) and recent studies suggested that representatives of the order Planctomyces are involved in the lithotrophic nitrification under anaerobic conditions (Strous *et al.* 1999). Under completely anoxic conditions ammonia is oxidized

with nitrite as an electron acceptor to nitrogen gas and small amounts of nitrate (Jetten *et al.* 1999).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O.$$

Hydroxylamine and hydrazine were identified as important intermediates. Anammox bacteria are extremely slow growing and are difficult to culture, and divides only once every two weeks (Strous *et al.* 1999a). Bioreactors with efficient biomass retention were used to obtain sufficient anammox biomass. Three known genera of anammox bacteria are *Brocadia, Kuenenia,* and *Scalindua* have been found in waste water treatment facilities and in marine ecosystems. Planctomycetes - like anammox bacteria were reported in waste water treatment facilities with limited oxygen supply and high nitrogen load (Van Dongen *et al.* 2001). Anammox bacteria are natural partners of aerobic ammonia-oxidizers in oxygen limited conditions and surveys revealed their presence in rotating biological contactors, trickling filters, biofilm reactors, fluidized bed reactors, sequencing batch reactors and even in fresh water wetlands (Jetten *et al.* 2002). Anammox bacteria play an important role in the nitrogen cycling of oceans (Kuypers *et al.* 2003).

1.2.5. Ammonia-oxidizing archaea (AOA)

A number of active ammonia-oxidizing archaea were discovered in agricultural and grassland soils (Treusch *et al.* 2005, Leininger *et al.* 2006). These ammonia-oxidizing archaea belongs to the phylum Crenarchaeota, possess ammonia monooxygenase (AMO) genes. The first ammonia-oxidising archeon, *Nitrosopumilus maritimus*, was isolated from a marine aquarium and shown to be chemolithoautotrophic capable of oxidizing ammonia to nitrite (Könneke *et al.* 2005). It also seems that it grows at similar rates and density as cultured AOB (Könneke *et al.* 2005).

1.2.6. Complete ammonia oxidizing bacteria (Comammox)

Based on the kinetic theory of optimal design of metabolic pathways, possible explanation for the division of labour in nitrification was proposed by Costa *et al.* (2006). They postulated the existence of optimal path length for a pathway that resulted in maximum ATP production rate. Shortening long pathways increased growth rate, but growth yields were low if the pathway had few ATP generating steps. In a typical biofilm high yields would be advantageous. It was postulated that bacteria that completely oxidize

ammonia to nitrate existed in biofilms (Costa *et al.* 2006). The longest possible nitrification pathway is of course, the complete oxidation of ammonia to nitrate. It was predicted that such a reaction would have lower maximal growth rate but a higher yield (Costa *et al.* 2006). The postulate of the existence of 'complete ammonia oxidizer' (comammox), was backed by the fact that in biofilms with a broad range of suitable conditions, an economical but slow growing organism would have a higher fitness than a resource wasting fast growing competitor (Kreft, 2004).

1.3. Denitrification

Denitrification is the reduction of nitrate to nitrogen gas involving many intermediates like HNO_2, NO and N_2O. It is a dissimilatory reduction of ionic nitrogen oxides (nitrate and nitrite) by aerobic bacteria, to the gaseous oxides like nitric oxide (NO) and nitrous oxide (N_2O), which may themselves be further reduced to nitrogen gas (N_2) (Knowles, 1982). It is the mechanism by which the global nitrogen cycle is balanced, it is responsible for the reduction of nitrogen oxides from the agricultural land, ultimate mechanism for nitrogen removal from waste and contribute nitrous oxide which is a concern in ozone depletion. Biochemically and taxonomically diverse bacteria are able to use nitrogen oxides as electron acceptors instead of oxygen. Most are heterotrophs, some utilize one-carbon compounds, some are autotrophic using H_2, CO_2 and reduced sulphur compounds and some others are photosynthetic (Knowles, 1982). The availability of electrons in organic carbon compounds is one of the most important factors controlling the activity of heterotrophs, comprising the bulk of denitrifiers.

In denitrification nitrate is reduced to dinitrogen gas by four reaction steps, mediated by four different metalloenzymes: nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*) and nitrous oxide reductase (*nos*). These enzymes are sequentially activated by anaerobic conditions (Philippot, 2002).

$$NO_3^- \xrightarrow[Nar]{} NO_2^- \xrightarrow[Nir]{} NO \xrightarrow[Nor]{} N_2O \xrightarrow[Nos]{} N_2$$

Development of cost-effective, integrated nitrogen removal system including both nitrification and denitrification process is essential for sustainable recirculation system, and only such a system will pave path for the advent of truly closed recirculating aquaculture system (Lee *et al.* 2000).

1.4. State of the art - Nitrification in aquaculture

1.4.1. Biological filters

Biological filters can be broadly classified into suspended growth and fixed film types. Biological nitrification can be carried out using either suspended growth reactors (activated sludge process) or fixed biofilm/ attached growth reactors. Extensive documentation is available for nitrification with different types of biofilters based on fixed biofilm technology in aquaculture. Recirculating aquaculture industry was inclined to use fixed film bioreactors rather than suspended growth systems (Malone and Pfeiffer, 2006). In aquaculture, nitrification is carried out in six general types: submerged filters, trickling filters, reciprocating filters, rotating biological contactors, rotating drums, and fluidized bed reactors (Rijn, 1996). Biofilter design guidelines have three main strategies, surface area, substrate transport across biofilm and biofilm management (Golz, 1995).

1.4.1.1. Suspended growth reactors

In suspended growth reactors, wastewater is brought into contact with microbial population in the form of a flocculant suspension under heavy aeration. This process more popularly known as activated sludge process has been particularly useful in sewage treatment facility treating waste water with high biodegradable waste loading. The activated sludge process is one of the most widespread biological wastewater purification technologies. In this process, wastewater is mixed with a concentrated bacterial biomass suspension (the activated sludge) which degrades the pollutants. Suspended and colloidal particles are readily adsorbed to the microbial flocs and dissolved nutrients are readily absorbed. Thus complex organic compounds are broken down by microbial metabolism and oxidized to simpler products like carbon dioxide. To develop a stable autotrophic bacterial population, a portion of separated sludge should be continually recycled to the aeration tank, and this requires continuous monitoring. An operational requirement of suspended growth reactor makes it a large scale process which demands intensive operational management. The sludge produced is removed and disposal of sludge is a problem associated with this process. Originally, the concern was mainly to remove the organic carbon substances from the wastewater, which could be obtained rather easily by simple process designs. However, more stringent effluent standards for nutrients (nitrogen and phosphorus) have been imposed by statutes and authorities. As a consequence, the

design and operation of activated sludge plants had to be modified to more advanced levels to make the treatment plants suited for biological nitrogen and phosphorus removal.

1.4.1.2. Fixed biofilm filters

Fixed film bioreactors are widely used in intensive aquaculture systems and hatcheries. They are regarded as appropriate technology for aquaculture industry where good water quality is a secondary objective to the production of large quantities of fish and crustaceans (Malone and Pfeiffer, 2006). Optimizing the parameters for biological nitrogen removal filters in recirculated aquaculture system is an important research area in modern aquaculture (Tal *et al.* 2003). Trend of examining biological filtration systems used in aquaculture has begun recently, particularly the microbial diversity and population structure in biofilm using tools like FISH and DGGE (Mertoglu *et al.* 2005). Very little information is available about the specific members that constitute the bacterial consortia in nitrifying filters. Such filters often contain anaerobic regions and involvement of anaerobic ammonia oxidizing (anammox) bacteria in the nitrogen removal process, which has to be examined. Dissolved and colloidal wastes enter into the biofilm by diffusion and undergo the mineralization process mediated by microbial action. Biofilm which coats the filter media like rocks, shells, sand and plastics can be regarded as a bacterial habitat which retains the inherent ability to process waste in different conditions. Fixed film biofilters were grouped as four fundamental types based on the strategy to provide oxygen and by their means to handle excessive biofilm growth (Malone and Pfeiffer, 2006).

There are submerged and emergent types of fixed film bioreactors, and submerged type is further grouped as packed, expandable and expanded (Malone and Pfeiffer, 2006). Rotating biological contactors and trickling filters are important emergent biological filter types. Submerged packed fixed film filter has three main versions, submerged rock, plastic packed bed and shell filters. Packed beds provide no active means of biofilm removal and they depend entirely on endogenous respiration for controlling biofilm accumulation (Manthe *et al.* 1988). Up-flow sand filters, floating bead bioclarifier and foam filters come under expandable submerged fixed film technology. The biofilm formed in the static bed can be removed periodically by abrasion, random rubbing and striking of individual medium with each other, which is often induced by hydraulic, pneumatic or mechanical force. In expanded submerged filters the biofilm is always abraded due to the constant motion of the bed. Expanded submerged fixed film bioreactors include fluidized sand,

down flow micro-bead and moving bed reactor (Malone and Pfeiffer, 2006). Fluidized filters are the oldest and most versatile in terms of choice of media types and size.

Fixed film bioreactors have their own sets of limitations. Biofilm itself is the result of rapidly growing heterotrophic bacteria, while performance of filter is usually determined by sensitive chemoautotrophic nitrifiers. High organic loading invariably increase heterotrophic bacterial growth, which in turn increase the biofilm thickness and decrease the nutrient diffusion into biofilm. This diffusional limitation on nutrient supply and controlling excessive biofilm development were the factors which hampers the overall performance of such biological filters.

Emergent filter types are designed to maximize oxygen transfer. Water is sprayed or cascaded directly over the media; in trickling filter, water is sprayed over the media by a rotating arm with a series of sprayers and nozzles. Whereas in rotating biological contactors/ drums media is rotated in and out of the water. Excessive biofilm which often results in clogging and fouling is managed by the process of sloughing. The sloughing process demands a relatively high porosity and high interstitial distances, for easy removal of excessive biofilm. Biodrums utilizes a loosely held media enclosed in a rotating drum (Wortman and Wheaton, 1991), this improves the biofilm sloughing process. Rotating biological contactors were found to give the best performance with respect to specific ammonia removal efficiency. But the use of RBC has been limited due to the mechanical problems related to shaft and bearings.

Trickling filters consist of a fixed media bed through which particle free waste water trickles down across the height of trickling filter (Eding *et al.* 2006). Oxygen is supplied from the bottom which oxygenates the whole bed and vent out carbon dioxide produced. The main disadvantages are low volumetric removal rates, biofilm shedding and risk of clogging due to excessive biofilm formation (Eding *et al.* 2006).

Submerged filters rely on high water circulation for their oxygen supply. Submerged types can be easily integrated with an intensive recirculation system with high water recirculation rates, internal recycling or through oxygen enrichment of influent waters. These filters are further grouped based on the biofilm control mechanism.

Biofilters are appropriate technology to control water quality in intensive shrimp culture systems which operate through out the year. Biofilters are used to sustain satisfactory water quality in intensive recirculating aquaculture ponds (Gross *et al.* 2003). However, high capital cost, operational and maintenance cost of biofiltration technology makes it a least preferred means to control water quality when it comes to small scale and

seasonal shrimp farmers. Another important fact is the limitations imposed by several formats of biofilters on the organic load of the influent water. Heterotrophs which grow rapidly at high organic load out-compete other more significant groups of chemoautotrophic bacteria like nitrifiers. Thus nitrification efficiency depends on the organic load of influent water and several new formats of biofilters like continuous circulating bed reactor (Nogueira *et al.* 2002) have been reported to counter the excessive heterotrophic growth. Addition of organic carbon drastically reduces biofilter's performance with long hydraulic retention time (Nogueira *et al.* 2002). FISH analysis of biofilm from such biofilm reactors revealed unexpected heterotrophic growth on top of the biofilm limiting oxygen supply to nitrifiers (Nogueira *et al.* 2002). Less intensive shrimp culture systems operate with intermittent shut down periods and biofilters are hard to maintain under such non-continuous culture practice. Above all, operation and maintenance of biofilters demands a fair level of expertise and knowledge. Trickling filters are notorious for clogging due to excessive biofilm formation and particulates in the feed water. They also demand additional land and capital requirements like power backup facilities. Rotating biological contactors/ drums experience frequent failure due to mechanical problems and they are fairly power intensive, and demands power backup for optimal performance. Submerged biofilters/ reactors need huge capital investment and should be monitored closely for efficient performance. But the prime factor which make these technologies away from small scale shrimp farmers, are the capital investment followed by the operational and fairly intensive maintenance cost involved. Biofilters commonly used today are very inefficient when very low levels of ammonia are required. Such low ammonia concentrations can be achieved if an array of different types of biofilteration technologies is employed simultaneously. However, this option will not be encouraged by small scale and seasonal shrimp farmers, due to the capital cost involved.

1.5. Hydrogen sulphide production and toxicity in aquaculture

Hydrogen sulphide is produced naturally in an aquaculture system due to anaerobic decomposition of organic matter and by the action of sulphate reducing bacteria. In sediments, sulphate and H_2S are constantly recycled between oxidation and reduction step, due to microbial action. Oxidation of H_2S is very important in ponds, which otherwise will lead to blackening of sediment and from sediments it will diffuse into overlying water column, triggering deleterious reactions. Recommended safe hydrogen sulphide concentration in shrimp culture is less than 0.02mg/L, preferably not detectable

(Whetstone *et.al.*, 2002). Exposure to H_2S is extremely toxic to aquatic fauna, it may affect health, survival and productivity of aquatic organisms. H_2S readily diffuses across membranes and inhibit respiratory enzymes and blood pigments (Phillips *et al.* 1997). H_2S is recognized as a potent inhibitor of cytochrome *c* oxidase, the terminal enzyme of oxidative phosphorylation. This involves the binding of the heme of the enzyme, with the greatest affinity being for the oxidized (Fe^{3+}) state. This process completely inhibits the aerobic metabolism, followed by ATP depletion (Eghbal *et al.* 2004).

1.5.1. Control of sulphide toxicity in aquaculture

Control of sulfide concentration in aquaculture systems can be effectively attained by either of the two following approaches: (i) increase of the redox potential in the pond by chemical poising (typically oxygen or nitrate). This has the effect of minimizing sulfate reducing bacteria activity and thus sulfide formation. However, preventing the formation of anaerobic conditions in sediments usually requires maintaining a relatively high oxygen or nitrate concentration in the bulk water above the sediment. This is possible but at a considerable cost. (ii) Removal of the sulfide after its formation, by either chemical oxidation or precipitation of insoluble metal sulfides, or a combination of both. Strong oxidizing agents such as H_2O_2 or $KMnO_3$ that are sometimes used to reduce sulfide concentration in sewers cannot be utilized in aquaculture systems. The addition of soluble ferric ions or amorphous ferric species (eg: ferrihydrite) as oxidizing agents has been used successfully for reducing sulfide effects in sewer drains, but in aquaculture ponds such application would not appear to be cost effective, because of the high cost of the chemicals accompanied with low regeneration kinetics (Lahav *et al.* 2004). Use of sulphide oxidizing green and purple sulphur bacteria and suitable means of incorporating such bioremediation technology is an environment benign approach and such an option has raised considerable interest in aquaculture sector.

1.6. Objectives.

1) Resolution of nitrifying bacterial consortia used in nitrifying bioreactors, and their characterization.
2) Development of novel bioaugmentors, nitrifying consortia immobilized on wood powder for amelioration of ammonia in shrimp culture systems.
3) Development of photosynthetic purple and green sulphur bacterial enrichment consortia for the bio-oxidation of hydrogen sulphide in shrimp culture systems.

1.7. Scope of the thesis.

Objective of the thesis is to propose a sustainable, low cost option for the mitigation of toxic ammonia and hydrogen sulphide in shrimp culture systems. Use of 'bioaugmentors' as pond additives is an emerging field in aquaculture. Understanding the role of organisms involved in the 'bioaugmentor' will obviously help to optimize conditions for their activity. Though the classical approach of enrichment and isolation is laborious, it is important to gain some insight on the actual diversity of species involved in the process and to define their eco-physiological properties. Though several molecular methods are useful for in situ analysis of nitrifying bacterial populations, complete molecular data are available only with those species that are in culture. This work is an attempt to define nitrifying consortia which are used as start up culture for novel bioreactor technology and as putative bioaugmentors, by resolving the individual components constituting the consortia and assessing their contribution to nitrifying activity.

Commercial nitrifying preparations are least studied on their ability to control TAN loadings under continuous TAN production. Immobilized nitrifiers tend to perform better and circumvent their notorious attributes like slow growth rate and high sensitivity to adverse conditions. The thesis describes the use of wood powder as carrier material for the immobilization of nitrifying consortia.

H_2S is a toxic pollutant ranked as one of the most important inhibitors of aerobic respiration. H_2S generated in aquaculture systems may cause plethora of problems due to its corrosive property, toxicity and its heavy oxygen demand. Conventional methods commonly adopted for sulphide oxidation are based on physico-chemical processes. H_2S removal by microbiological methods especially by photosynthetic sulphur bacteria is a good alternative to physico-chemical treatments because of its economic feasibility. This strategy is yet to be adopted in aquaculture industry.

Chapter 2

Resolution of nitrifying bacterial consortia and characterization.

2.1. Introduction

Two ammonia oxidizing (AMONPCU-1 and AMOPCU-1) and two nitrite oxidizing (NIONPCU-1 and NIOPCU-1) bacterial consortia for activating nitrifying bioreactors and thereby establishing nitrification in penaeid and non-penaeid hatchery systems were developed by enrichment (Achuthan *et al.* 2006). A protocol for the amplification of the nitrifying bacterial consortia in simple seawater based medium (salinity 15 or 30) supplemented with NH_4^+-N or NO_2^--N and PO_4^--P with pH 8.0 in a fermentor, was developed. During amplification the consortia exhibited excessive wall growth and diminished their yield coefficient posing difficulty in complete recovery of the biomass generated. The consortia consisted of both Gram negative and Gram positive bacterial cells embedded in mucilaginous matrix of glycocalyx – like material presumably composed of polysaccharides. The consortia besides being useful in activating nitrifying bioreactors developed for shrimp/prawn hatchery systems can also be used as bioaugmentors in the bioremediation of ammonia and nitrite toxicity in aquaculture systems (Achuthan *et al.* 2006). For the development of an appropriate bioremediation for shrimp grow out system, these consortia were adopted. As the prime step, phenotypic and genotypic characterization of these consortia was attempted.

A review of the efforts taken globally to study such nitrifying bacterial consortia revealed the following:

In nature, most of the ecological niches are occupied by specific microbial communities (Puzyr *et al.* 2001). Consortia are quasi stable interacting mixtures of diverse strains, individual strains of microbial cells and intra-cellular macromolecules (Hamer, 1997). Microbial consortia are widely used as bioaugmentors for the treatment of environmental pollutants. A fundamental problem in assessing both microbial resources and their metabolic potential in biological process systems is the inability to either isolate or characterize most of the microorganisms present in such systems. This in fact hinder the continuing development of microbe mediated treatments as the data regarding the physiology of microorganisms present or used in biotreatment process cultures remain obscure (Hamer, 1997). It has to be highlighted that modification of inorganic dissolved nitrogen by nitrifying bacteria is an important objective of all waste water treatment processes (Hamer, 1997) and aquaculture/ aquaria operations (Grommen *et al.* 2002). The responsible bacteria were considered to be a chemolithotrophic consortium of *Nitrosomonas* and *Nitrobacter;* and any possible role of chemoorganotrophic (heterotrophic) nitrifying bacteria was grossly ignored (Hamer, 1997). But nitrifiers in

contaminated cultures have been demonstrated to nitrify more efficiently, possibly due to the association of chemoorgantrophic bacteria. In principle, understanding microbial community structure and functioning should be a major objective in the design and operation of treatment systems (Yuan and Blackall, 2002). Even though breakthroughs in reactor designs along with monitoring of physico-chemical variables helps to enhance and optimize biological reactions, consistent long term performance can only be ensured when the microbial community in the sludge/ biofilm functions optimally (Geets *et al.* 2007). Both ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) have prolonged generation time and usually takes several months to obtain pure cultures. Puzyr *et al.* (2001) attempted to define nitrifying consortia by resolving the individual components constituting the consortia using light and electron microscopy and attempted an assessment on their contribution to nitrifying activity. In this study, spatial location of different cells, distribution of different cell type, with respect to the nitrogen substrate were revealed and this approach was proposed as a method to study microbial associations and communities (Puzyr *et al.* 2001).

Nitrification is an important and inevitable factor in the global nitrogen cycle and in situ studies on natural bacterial populations participating in this process has increased considerably (Koops and Pommerening-Röser, 2001). The study of nitrifying bacteria known to play an important part in nitrogen cycle, is very much a herculian task due to their slow growth and very small colony size (less than 100μm in diamcter). The severe problems encountered in isolating pure cultures of nitrifying bacteria offer an explanation for the fact that only a very limited number of strains of these microorganisms have been studied to date (Puzyr *et al.* 2001). Presently very few species of AOB and NOB are cultured but existence of many more species has been proved by molecular in situ studies (Koops and Pommerening-Röser, 2001). Therefore, what we know is a fraction of existing nitrifiers isolated and defined by physiological and molecular characterization.

Nitrifiers, along with denitrifiers strongly contribute to the global nitrogen cycle and hence it is important to study their distribution and diversity. Composition of nitrifying bacterial communities is complex and diverse in different habitats due to dependence of distribution pattern on various environmental parameters. For this reason, nitrifying community structure elucidated only by in situ studies would be incomplete and unbalanced. Therefore, a combination of classical and molecular methods in parallel would help overcome several hurdles for characterizing natural nitrifying bacterial

communities. Isolation and characterization of as many as new species seems to be the most important missing step in this endeavour (Koops and Pommerening-Röser, 2001). The most common classical method to enumerate culturable nitrifiers is the MPN (most probable number) technique, which grossly underestimates the cell counts, since nitrifiers often occur as cell aggregates (zoogloea) in natural habitats (Koops and Pommerening-Röser, 2001). Cell growth in autotrophic nitrifiers is very slow as it requires a lot of energy for metabolism and is left with little for cell multiplication. Because of this slow growth, the most practical methods of their enumeration, i.e., MPN count requires a long incubation period. According to Matulewich *et al.* (1975) maximum most probable number of ammonium-oxidizers was attained in 20-55 days and that of nitrite oxidizers in 103-113 days. Immunological analysis of nitrifying populations using the immunofluorescence is another method (Belser and Schmidt, 1978), but antigenic variations are common within natural nitrifying populations. Monoclonal antibodies targeting different subunits of the enzyme nitrite oxidoreductase were demonstrated to detect NOB to genus level (Bartosch *et al.* 1999).

Use of culture enrichment and culture-dependent molecular methods were employed to identify novel AOB in nitrifying freshwater aquarium (Burrell *et al.* 2001). Though several molecular methods are useful for in situ analysis of nitrifying bacterial populations, complete molecular data are available only with those species that are in culture (Koops and Pommerening-Röser, 2001). FISH technique has been effective for the rapid enumeration and can give significant insight on the nitrifying microbial community both in environmental samples and in biological nitrification reactors (Chae *et al.* 2007). In the review by Koops and Pommerening-Röser, (2001) it was opined that extrapolation is not possible from relative quantities of definable DNA to absolute cell counts; since cellular DNA quantity is not stable due to shifts in metabolic trends. There is only a limited degree of 16S rDNA sequence diversity within environmental nitrifiers sequence clusters due to the close relationship among the involved species. Therefore, in many cases it cannot be stated with certainty how many distinct species are represented by the obtained sequences or whether it indicates the presence of any yet uncultured species (Koops and Pommerening-Röser, 2001). Molecular analysis targeting DNA can provide some measure of the presence of particular bacterial groups in a population and give little information on the activity (Kowalchuk and Stephen, 2001). The amount of rRNA within a cell is thought to be the best approximation of cellular activity, and techniques like RT-PCR, in situ hybridization coupled with image analysis would give some valuable data on

activity regimes. Since nitrifiers are slow growers they hardly emerge as the dominant species in most environments and this fact emphasize that analysis with rRNA genes are less effective (Tamegai *et al.* 2007). Microsensor measurement is a useful tool to link chemical profiles in relation to the distribution of different bacterial taxa, it may be used not only to identify environmental conditions favoured by particular bacteria, but can also be used to quantify specific bacterial mediated processes (Gray and Head, 2001).

Five genera of ammonia oxidizers have been reported namely *Nitrosomonas, Nitrosospira, Nitrosolobus, Nitrosovibrio* and *Nitrosococcus*. Four genera of nitrite oxidizers are *Nitrobacter, Nitrospina, Nitrococcus* and *Nitrospira*. In contrast to their common physiology, a high degree of phylogenetic diversity was found among the AOB and NOB. These organisms are scattered among α, β, γ, and δ subclasses of Proteobacteria; the genus *Nitrospira* (NOB) occupy a phylogenetically isolated position in a separate phylum (Spieck and Bock, 2002). AOB are restricted to two distinct lineages within the Proteobacteria. All AOB form a closely related monophyletic cluster within the β-Proteobacteria, except *Nitrosococcus*, which forms a separate branch within the γ-Proteobacteria. NOB belongs to α and γ subclass of Proteobacteria, the genus *Nitrospina* belongs to a separate group with yet uncertain placement (Costa *et al.* 2006).

The nitrifying bacterial consortia (NBC) used in this study were the ones generated seven years back and had been under repeated amplification and storage. However, they were not characterized and at this stage as they were to be used as bioaugmentors it was essential to be analyzed. This chapter describes the attempts made to resolve the bacteria involved in the consortia and to characterize them as the putative bioaugmentors in shrimp grow out systems.

2.2. Materials and methods

2.2.1. Amplification of nitrifying bacterial consortia in fermentor

For accomplishing resolution and characterization, the consortia were essentially required to be amplified. The amplification was carried out in a 2L, BIOFLO 2000 fermentor (New Brunswick scientific, USA). Watson's medium (1965) (NH_4^+-N or NO_2^--N 10mg/L and PO_4^--P 2mg/L, pH 7.0 to 8.0) prepared in seawater 15 or 30 salinity was used. The fermentor vessel assembly with the medium was autoclaved at 10 lbs, for 10 minutes. As a prerequisite for amplification, pH was set between 7.0 to 8.0, temperature at 28°C and agitation set at 200rpm and aeration adjusted at 0.6L/minute. Amplification was

done by inoculating and incubating the fermentor vessel with 20mL consortium from the stock and a black cloth wrapping was used to incubate the fermentor vessel under obscurity. Once in every 24 hours the substrate uptake and product formation were monitored and depending on the requirement, the substrate was added through the substrate addition port, pH correction was by automated dispensing of sterile 1.5M Na_2CO_3. When the rate of substrate uptake and product formation declined, the culture was harvested and concentrated by centrifugation at 8000rpm (7000g) at 4°C in a refrigerated centrifuge (Remi Instruments, Bombay). The biomass obtained was washed with fresh medium and resuspended in the medium supplemented with 10ppm substrate (NH_4Cl/ KNO_2). The concentrated consortia were stored in tightly stoppered sterile glass bottles at 4°C with intermittent addition of the substrates. Analysis of TAN, NO_2^--N and NO_3^--N was done spectrophotometrically (Solorzano, 1969, Bendschneider and Robinson, 1952 and Strickland and Parsons, 1968). On attaining stationary phase, the culture was harvested and stored at 4°C in polypropylene bottles with intermittent addition of the substrates and adjustment of pH.

2.2.2. Resolution of nitrifying bacterial consortia

Resolution of the NBC was attempted following the conventional streak plate method. The plates were prepared in Watson's medium (1965) solidified with phytagel (1%) (Sigma chemical co., USA) prepared in aged seawater of respective salinity. 20mL aliquots of Watson's medium containing 1% phytagel (w/v) was autoclaved at 15lbs/15 minutes and poured into petri plates at a temperature around 60°C. Phytagel plates were preferred for purification as it was devoid of assimilable organic nutrients and its transparency, which helped micro-colonies of nitrifiers to be distinguishable. Plates were inoculated (0.2mL) by spread plate method and incubated in dark at 28°C in a humidity controlled environmental test chamber (Remi Instruments, Bombay). Colonies developed on the plates were streaked onto phytagel plates until pure cultures having uniform colony morphology were obtained on the plates. They were isolated and maintained on phytagel slants. As soon as visible growth was observed on the slants, the cultures were Gram stained.

2.2.3. Purification and stocking

All isolates were purified by repeatedly streaking on phytagel plates. The pure cultures were transferred to phytagel vials, sealed with wax and stored at 28°C in dark.

Parallel sets of cultures were stocked in screw capped tubes with liquid media (Watson's, 1965) and another set was maintained in the presence of a cryoprotectant at -80°C. For stocking at -80°C, to 5mL of overnight grown ZoBell's broth culture 1.6mL of autoclaved 60% (v/v) glycerol solution was added and thoroughly mixed with a vortex mixer. 1mL from this mixture was dispensed into sterile, screw capped freeze- proof vials and stored at -80°C (Brown, 2000).

2.2.4. Phenotypic characterization of the resolved isolates

The cultures were subjected for the phenotypic characterization such as Gram's reaction, spore staining (Shaeffer-Fulton method), acid fast staining, marine oxidation fermentation reaction, catalase, methyl red reaction, production of amylase, Voges Proskauer reaction (Barrit, 1936), Kovacs' cytochrome oxidase (Kovacs, 1956), production of gelatinase (Frazier, 1926), lipase (Harrigan, and Mc Cance, 1972), cellulase, chitinase (Holding and Collee, 1971), DNAase (Jefferies, *et al.* 1957), arginine dihydrolase (Thornley, 1960), β-galactosidase (ONPG), aesculin hydrolysis (Gemmell and Hodgkiss, 1964), oxidation of gluconate, phenylalanine deaminase, indole production (Cowan and Steel, 1965), nitrate reduction, nitrite reduction, citrate utilization along with motility and cellular morphology.

2.2.5. Clustering based on unweighted average linkage and identification of clusters

Based on the phenotypic characterization of pure cultures, data matrix was generated by coding the results obtained from the tests as '1' for positive, '0' for negative, and '9' for doubtful results. The data matrix prepared in 'Excel' spreadsheets (Microsoft Office package) was converted to proprietary matrix files by the programme NTedit, Version 1.1b (Applied Biostatistics Inc.) and rectangular data matrix generated was analysed by the programme NTSYSpc, Version 2.02i (Applied Biostatistics Inc.). Similarities were calculated by the simple matching coefficient using statistical module, sequential agglomerative hierarchical nested cluster method (SAHN) and clustering was achieved by unweighted pair-group method, arithmetic average (UPGMA).

2.2.6. Transmission electron microscopy (TEM) of nitrifying consortia and pure cultures and demonstration of the characteristics of nitrifiers

The nitrifying bacterial consortia amplified in the fermentor were subjected for transmission electron microscopy. An aliquot of 10mL each of the consortia, were

centrifuged at 8000rpm (5400g) in a refrigerated centrifuge (Remi Instruments, Bombay) for 15 minutes. The pellets were washed with sterile seawater of respective salinity and fixed in 2.5% gluteraldehyde prepared in sterile seawater at 4°C overnight. The pellets were washed and post fixed in 2% osmium tetroxide at 4°C for 2 hours. Subsequently, the bacterial suspensions were washed repeatedly with seawater and dehydrated. The dehydration was done through an acetone series of 70 - 100%, embedded in epoxy resin, sectioned and stained with lead citrate and uranyl acetate and examined under electron microscope (Morgagni 265-D, Netherlands).

2.2.7. Determination of nitrifying potency of the pure cultures

The isolates resolved from ammonia and nitrite oxidizing consortia were tested for nitrifying potency. Cultures were tested for nitrifying potential both under aerated and non-aerated conditions. Cultures were inoculated into 50mL aliquots of the medium according to Watson (1965) in 100mL flasks and incubated under obscurity for 1 week. One batch was incubated under aeration in a shaker and other static. Substrate uptake (NH_4^+-N / NO_2^-- N) and product formation (NO_2^- - N / NO_3^-- N) were monitored regularly by spectrophotometry.

2.2.8. Determination of denitrifying potency of the pure cultures

Nitrate and nitrite removal of the isolated cultures were measured in Watson's medium (1965) supplemented with 10ppm nitrate/nitrite (KNO_3/$NaNO_2$). Daily reduction in NO_3^--N and NO_2^--N concentration was estimated.

2.2.9. Molecular characterization

As a part of the molecular characterization of the resolved pure cultures, amplification and sequencing analysis of ammonia monooxygenase (*amo*) gene and nitrite reductase (*nir*) genes were performed.

2.2.9.1. DNA extraction and purification

a) For Gram negative bacteria

1.5mL overnight grown culture in ZoBell's broth was centrifuged (Eppendorf, Germany) at 10000rpm for 5 minutes (4°C), cells resuspended in 1mL TNE buffer [0.1M Tris-Cl (pH 8.0), 0.15M NaCl and 0.01M EDTA] and centrifuged for 5 minutes (10000rpm at 4°C). Cell pellet incubated with 500µL lysis buffer [0.05M Tris-Cl (pH 8.0),

0.1M NaCl, 0.05M EDTA, 2% SDS, 0.2% PVP and 0.1% β mercapto ethanol] and 20 µL proteinase K (20mg/mL) in a water bath for 1 hour at 37°C and 1-2 hours at 55°C. DNA was extracted by phenol-chloroform-isoamyl alcohol mixture (25:24:1) and centrifuged at 15000rpm for 15 minutes (4°C). Aqueous phase was collected and subjected for further extraction with chloroform isoamyl alcohol mixture (24:1). Aqueous phase obtained after centrifugation (15000rpm/ 15 minutes) was kept overnight (-20°C) after addition of 50 µL sodium acetate solution and 500 µL ice cold ethanol. Precipitated DNA was collected by centrifugation at 20000g (4°C) for 15 minutes, and washed with 70% ice cold ethanol and subsequently resuspended in milliQ water. DNA concentration and purity were assessed spectrophotometrically (Hitachi U-2800, Hitachi Corp., Japan).

b) For Gram positive bacteria

1.5mL 48hr grown culture in ZoBell's broth was centrifuged (Eppendorf, Germany) at 10000rpm for 5 minutes (4°C), cells resuspended in 495 µL TNE buffer [0.1M Tris-Cl (pH 8.0), 0.15M NaCl and 0.01M EDTA] and 5 µL lysozyme (0.2g/mL) followed by incubation at 30°C for 30 minutes. Samples were again incubated after adding 50 µL proteinase K (20mg/mL), followed by addition of 70 µL SDS (10% w/v), 180 µL 5M NaCl and 150 µL 10% CTAB-NaCl solution and incubated at 65°C for 20 minutes. Subsequent steps were same as described in the case of Gram negative bacteria.

2.2.9.2. Amplification of ammonia monooxygenase (*amo*) enzyme genes

The ammonia monooxygenase enzyme is a key enzyme involved in nitrification. This enzyme mediates the oxygenation of ammonia to hydroxylamine, which is the characteristic enzyme of chemolithotrophic AOB. The AMO of *N. europaea* is a membrane bound enzyme which was extensively studied in chemolithotrophs containing copper at its active site. AMO consists of three subunits encoded by three genes *amo*A, *amo*B and *amo*C (Tamegai *et al.* 2007). Since *amo* genes are so important in the first step of nitrification, these genes are useful markers for detecting AOB in environmental samples.

For the amplification of *amoA* genes *amoA*-1F- *amoA*-2R primer system (Rotthauwe *et al.* 1997) were used. PCR amplifications from selected pure cultures were performed in a total volume of 25µL containing 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 20mM Tris HCl, pH 8.8), 500µM each deoxyribonucleoside triphosphate, 1 µL of both forward and reverse primer (7.5pmol/ µL)

and 1 µL of template DNA (100ng/ µL). After the initial denaturation at 94°C for 5 minutes, 1U taq polymerase (New England Biolabs) was added at a holding temperature of 80°C. PCR reaction was performed (Thermal cycler: Eppendorf Mastercycler Personal) with the following thermal profile: 42 cycles consisting of denaturation step at 94°C for 1 minute, primer-annealing step at 56.8°C for 90sec and elongation was performed at 72°C for 90sec. Final cycle consisted of an extended elongation step lasting about 10 minutes at 72°C. The amplification products (10 µL) were analysed by electrophoresis on 1% (wt/vol) agarose (Sigma) gels. Expected PCR amplification product generated by the primer system is a 491 bp long sequence (Rotthauwe *et al.* 1997).

Primer	Position[a]	Primer sequence (5'-3')
amoA 1F	332-349	GGGGTTTCTACTGGTGGT (18)
amoA 2R	802-822	CCCCTC(G/T)G(G/C)AAAGCCTTCTTC (21)

[a] Positions in the open reading frame for the *amoA* gene sequence of *Nitrosomonas europaea.* Rotthauwe *et al.* (1997)

2.2.9.3. Presence of nitrite reductase (*nir*) enzyme genes

Denitrifiers are a physiological group of facultative anaerobes, which can switch from oxygen to oxides of nitrogen as terminal electron acceptors under anoxic conditions. Nitrite reductase is the key enzyme in the dissimilatory denitrification process which mediates the reduction of nitrite to NO. This step can be catalysed by the product of two different nitrite reductase genes, one copper containing product of *nirK* gene and the other containing cytochrome cd_1, product of *nirS* gene. Though they are structurally different, both enzymes are functionally and physiologically equivalent. Two genes seem to occur mutually exclusively in a given strain, but both types have been found in different strains of same species. *nirS* is more widely distributed and *nirK* is found in a wider range of physiological groups but found only in 30% of the denitrifiers studied so far (Braker *et al.* 1998).

For the amplification of *nirK* and *nirS* genes *nir*S1F- *nir*S6R and *nir*K1F- *nir*K5R primer systems (Braker *et al.* 1998) were used. PCR amplifications from selected pure cultures were performed in a total volume of 25µL containing 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 20mM Tris HCl, pH 8.8), 500µM

each deoxyribonucleoside triphosphate, 2.5 µL of both forward and reverse primer (10pmol/ µL), and 1 µL of template DNA (100ng/µL). After the initial denaturation at 94°C for 5 minutes, 1U taq polymerase (New England Biolabs) was added and touch down PCR was performed (Thermal cycler: Eppendorf Mastercycler Personal). Initial 10 cycles consisted a denaturation step at 94°C for 30sec, primer-annealing step at 45°C to 40°C (0.5°C decreased by every cycle) for 40sec and elongation was performed at 72°C for 1 minute. Additional 30 cycles were performed with annealing step performed at 43°C for 40sec and final incubation at 72°C for 10 minutes was performed. The amplification products (10 µL) were analysed by electrophoresis on 1% (wt/vol) agarose (Sigma) gels. Expected amplification product for *nir*S1F- *nir*S6R and *nirK1F*- *nir*K5R were 890bp and 514bp sequences respectively (Braker *et al.* 1998).

Primer	Position[a]	Primer Sequence (5'- 3')
nirK1F	526-542	GG (A/C)ATGGT(G/T)CC(C/G)TGGCA (17)
nirK5R	1023-1040	GCCTCGATCAG(A/G) TT(A/G)TGG (18)
nirS1F	763-780	CCTA(C/T) TGGCCGCC(A/G)CA(A/G)T (18)
nirS6R	1638-1653	CGTTGAACTT(A/G) CCGGT (16)

[a] Positions in the nir K gene of *Alcaligenes faecalis* S-6 EMBL D13155 and in the nirS gene of *Pseudomonas stutzeri* ZoBell EMBL X56813.

2.3. Results

2.3.1. Amplification of consortia

Amplification of nitrifying consortia was carried out by transferring 1% (v/v) nitrifying bacterial consortia stored at 4°C into 2 L Watson's (1965) medium supplemented with 10ppm TAN/ nitrite (NH_4Cl/ $NaNO_2$) with continuous aeration. After one week of incubation under obscurity, average TAN consumptions were 6.57 ± 0.68 mg/L (AMONPCU-1) and 6.78 ± 1.69 mg/L (AMOPCU-1). In the case of nitrite oxidizing bacterial consortia, substrate consumption was 5.74 ± 1.04 mg/L (NIONPCU-1) and 6.41 ± 1.43 mg/L (NIOPCU-1). In all amplification trials with all the nitrifying bacterial consortia, product (NO_2^--N/ NO_3^--N) formation was very low or below detectable limit (Table 2.1).

2.3.2. Resolution of NBC on phytagel plates and determination of heterotrophic growth

A total of 96 cultures were isolated and purified from all four nitrifying bacterial consortia (AMONPCU-1, AMOPCU-1, NIONPCU-1 and NIOPCU-1). All the isolated cultures were found to grow on ZoBell's agar plates, indicating heterotrophic growth. From the consortium AMONPCU-1, 18 pure cultures were obtained and they were grouped into three, as shown in Table (2.2), based on colony morphology on phytagel plates, Gram's reaction and cellular morphology. All of them exhibited heterotrophic growth. Group 1 which included majority of the isolates, were actinomycete-like in colony morphology and Gram positive rods. Two other minor groups were composed of transparent, irregular, lobate, flat colonies in which one group was composed of Gram positive and the other Gram negative. From the consortium AMOPCU-1, 15 isolates were obtained (Table 2.3) and they formed three groups. The dominant one was whitish, irregular, lobate and flat, Gram negative rods and the other one whitish, pinpoint, entire, flat, Gram positive rods and the third group, grey, circular, erose, umbonate, actinomycetes like Gram positive rods. All of them showed heterotrophic growth.

A total of 48 isolates were obtained from the consortium NIONPCU-1 (Table 2.4). Majority of the isolates were actinomycetes like Gram positive rods. Besides Gram negative rods, Gram positive and Gram negative cocci could also be resolved with distinct colony morphology. From the consortium NIOPCU-1, 15 isolates were obtained (Table 2.5). Both Gram positive and Gram negative rods and Gram positive cocci could be resolved having a variety of colony morphology.

2.3.3. Phenotypic characteristics of the resolved isolates

Results of biochemical characterization of the pure cultures isolated from AMONPCU-1, AMOPCU-1, NIONPCU-1 and NIOPCU-1 are summarized in Tables 2.6, 2.7, 2.8 and 2.9 respectively.

2.3.4. Clustering based on unweighted average linkage and identification of the clusters

Based on the biochemical profile, cultures resolved from each consortia were clustered using the programme NTSYSpc (Version 2.02i). Dendrograms (Fig 2.1, 2.2, 2.3 and 2.4) were generated based on the similarity index. Clustering of the cultures was achieved at > 80 % similarity based on the phenotypic characters examined. Accordingly, AMONPCU-1, AMOPCU-1, NIONPCU-1 and NIOPCU-1 exhibited 9, 8, 17 and 8 clusters respectively.

2.3.5. Transmission electron microscopy of the NBC and pure cultures and demonstration of the characteristics of nitrifiers

Ultrastructure of ammonia oxidizing consortia such as AMONPCU – 1 and AMOPCU – 1 and the pure cultures obtained by way of resolution are presented in Fig. 2.5 and 2.6 respectively. In ammonia oxidizing consortia (Fig. 2.5 and 2.6), cells were rod shaped, spherical or lobular without endospores. Some, but not all, possessed characteristic intracytoplasmic membranes as flattened lamellae arranged centrally, peripherally or randomly. Carboxysomes and/ or polyphosphate like inclusions were observed in some cells. Most cells had extra cellular polymeric substance (EPS) like envelope. Cells were also found embedded in slime forming aggregates called cysts (Fig. 2.5a & 2.6a). Four different types of cells were observed on analyzing the ultrastructure of cells in ammonia oxidizing consortia.

Type 1: Spherical, with intracytoplasmic membranes, due to the infoldings of the plasma membrane

Type 2: Cytomembranes absent.

Type 3: Cytomembranes randomly arranged throughout the cytoplasm.

Type 4: Cytomembranes in the form of a polar cap of flattened vesicles in the peripheral region of the cell, cytoplasmic inclusions present.

Ultrastructure of ammonia oxidizing consortia such as NIONPCU – 1 and NIOPCU – 1 and the pure cultures obtained by way of resolution are presented in Fig. 2.7 and 2.8 respectively. The cells were rod to pear shaped and spherical without endospores. Some possessed intracytoplasmic membranes which occurred as flattened lamellae or tubular structures. Cells were aggregated to form cysts and zoogloea (Fig. 2.7c and 2.8a). An interesting observation was the presence of curved cells (Fig. 2.7b) like those of *Nitrosovibrio* without extensive cytomembranes, characteristic of nitrite oxidizing bacteria.

In general, transmission electron microscopy revealed the presence of peripherally arranged intracytoplasmic membranes in both consortia and a few pure cultures examined. Several layers of intracytoplasmic membranes were clear in Fig. 2.8d.

2.3.6. Determination of nitrifying potency of the pure cultures

Isolated cultures were tested for their nitrifying potency under both aerated and non aerated conditions. When cultures were incubated under obscurity with out aeration, nitrification was not detected even after incubating for more than one month. But

nitrification could be detected in cultures maintained with aeration on a rotating shaker under obscurity, within 1 to 7 days. All cultures exhibited nitrification potential under aerated condition. Nitrifying potency of pure cultures isolated from AMONPCU-1, AMOPCU-1, NIONPCU-1 and NIOPCU-1 is tabulated in Table 2.10 and Table 2.11.

All the 33 pure isolates obtained from AMONPCU-1 and NIONPCU-1 exhibited the two stage nitrifying potency (oxidation of ammonia to nitrate) and in a few cases nitrite could not be detected and instead nitrate could be detected. Out of 63 pure isolates from NIONPCU-1 and NIOPCU-1, six cultures did not show nitrate build up while all others could generate nitrate from nitrite.

2.3.7. Determination of denitrifying potency of the pure cultures

Isolated cultures were tested for their denitrifying potency by measuring nitrate consumption and subsequent nitrite production in liquid media containing 0.1% (w/v) KNO_3 and results are tabulated in Table 2.12. Majority of the isolates from all nitrifying bacterial consortia exhibited nitrate reduction. A comparison of denitrifying and nitrifying potency of all the pure cultures is given in Table 2.13.

2.3.8. Molecular characterization

The pure cultures segregated representing each of the clusters in the dendrogram for PCR amplification of the genes encoding *amoA* and *nirK* & *nirS* are listed in Table 2.14. Amplifications obtained based on the primers for *amoA* gene are presented in Fig. 2.9. The amplifications in the expected band range of 491bp were obtained for the isolates N29, N30 and N83. It has to be pointed out that N83 belongs to NIOPCU-1 the nitrite oxidizing consortium. The PCR products of N32 and N92 showed a 100 bp band shift from the expected amplicon. This suggests the requirement of more standardization of the PCR. Meanwhile, it has to be realized that such band shifts are not unusual in wild isolates.

Amplifications obtained based on the primers of *nirS* are presented in Fig. 2.10. In fourteen isolates such as N11, N15, N18, N24, N25, N29, N30, N32, N39, N47, N72, N83, N88 and N92 putative *nirS* gene could be amplified with a band shift in N11, N15, N32, N47 and N83.

Amplifications obtained based on the primers of *nirK* are presented in Fig. 2. 11. In eight isolates such as N21, N24, N29, N30, N 32, N66, N82 and N88 putative *nirK* gene could be amplified with a band shift in N21, N24, N29, N30, N66 and N82.

2. 4. Discussion

For characterizing the NBC maintained at 4°C, they were amplified in a fermentor initially. During the amplification it was observed that the products of nitrification (NO_2^--N and NO_3^- -N) were extremely low, however, with substantial substrate (NH_4^+ -N and NO_2^-- N) uptake. This has been the observation through out the study period. Suspecting that the organisms do involve in denitrification also, the resolved pure isolates were examined for denitrifying potency. While doing so majority of them were found reduce NO_3^-- N to NO_2^-- N. Several of them could even reduce NO_2^-- N further. This answered the question why the products of nitrification in fermentor were detected at very low concentrations. A similar observation was made by Du *et al.* (2003) on studying a mixed culture of nitrifying and denitrifying bacteria for simultaneous nitrification and denitrification. Ammonia could be oxidized aerobically to nitrate by the mixed culture and the intermediate NO_2^-- N was then reduced to dinitrogen gas. No nitrite was detected during the process. However, in the present study nitrite and nitrate were detected as products of nitrification but in very low concentration and not found being built up. Presumably, they might be getting reduced to either dinitrogen or ammonia. However, chances of ammonia build up were ruled out as ammonia uptake was substantial during the experiment. Therefore it was concluded that the consortia oxidized ammonia to either nitrite or nitrate and from there to dinitrogen. This has been suggested as an ideal situation as far as the aquaculture systems are concerned as removal of ammonia to dinitrogen is the process highly favourable for the cultured stock.

A total of 96 pure cultures were generated from all four consortia all together. All the isolates exhibited heterotrophic growth. Previous literature suggests that some but not all nitrite oxidizers can grow mixotrophically in a medium supplemented with yeast extract and peptone as nitrogen sources and pyruvate or acetate as the carbon source. When they were grown mixotrophically, the generation time decreased, and the cell yield went up by a factor of 10. Only *Nitrobacter* species were able to grow heterotrophically (Watson *et al.* 1989). Majority of the pure cultures obtained from the NBC in the study were Gram positive, either rods or cocci, with actinomycetes colony morphology. Out of 96 isolates only 25 were Gram negative. As on date, Gram positive autotrophic nitrifiers have not been reported. However, in the consortia under investigation they are the dominant forms. According to Watson *et al.* (1989), most, but not all nitrifiers, have a typical Gram negative multilayered cell wall. They do nitrify with the formation of nitrite, the product of autotrophic nitrification, a specialty of nitrifiers. This has not been hitherto

reported in heterotrophic nitrifiers. Therefore, the cultures resolved from NBC which exhibited heterotrophic growth can not be nomenclatured as heterotrophic nitrifiers at this stage since more investigations are required for their delineation. It is uncertain whether heterotrophic nitrification is via an inorganic pathway involving HAO and nitrite or via an organic pathway involving oxidation of amine or amide to a substituted hydroxylamine and with subsequent oxidation to nitroso and then to a nitro compound (Nugroho, 2006) or it may be a combined inorganic and organic pathway (Killham, 1990).

Considering the heterotrophic growth, all the isolates were subjected for biochemical characterization and clustering based on unweighted average linkage. Several distinct clusters (42) having 80% and above similarity could be differentiated. This treatment helped in selecting representatives for molecular level analysis of the genes encoding *amoA, nirS* and *nirK*. However, with the phenotypic characteristics investigated the clusters could not be identified.

On examining the ultra structural details of the consortia and cells, characteristic features of nitrifiers such as intracytoplasmic membranes, carboxysomes, polyphosphate like inclusions, EPS, cysts, zoogloea, curved rods, etc could be demonstrated. All these characteristics have been documented as unique features of autotrophic nitrifiers (Watson *et al.* 1989). This observation assumes very important dimensions in the light of evidence of heterotrophic growth of the isolates. Therefore, a judgment on their status as either heterotrophic nitrifiers or autotrophic nitrifiers has to be made based on these evidences. Carboxysomes occur in all *Nitrobacter* strains grown chemolithotrophically but rarely occur in heterotrophically grown cells. Heterotrophically grown cells often lack or have a reduced number of intra-cytoplasmic membranes (Watson *et al.* 1989).

All the 96 pure isolates from the consortia exhibited nitrifying potency, when they were incubated under obscurity with aeration and agitation. The requirements of aeration and agitation have been observed with the consortia for efficient nitrification through out the experiment. This can be taken for granted as a specific requirement for nitrification as mass transfer is the key factor for efficient oxidative processes in general.

One of the most interesting and important observations with respect to the pure cultures resolved from the consortia is their denitrifying potency, exhibited as either as nitrate or nitrite reduction. Among them 47 isolates exhibited both the properties. Both nitrifying and denitrifying properties were exhibited by 90 isolates which could be considered as the most unique feature of the consortia as the organisms in them were able to convert ammonia nitrogen or nitrite nitrogen to the products of denitrification through nitrification.

This is a complex situation not reported earlier. However, genes involved in denitrification (*nirK* and *norB*) have been annotated on the *N. europaea* ATCC 19718 genome and detected by PCR amplification in pure cultures of marine *Nitrosomonas* and *Nitrosococcus sp.* (Shaw *et al.* 2006). No bacterial species which oxidizes ammonia to nitrate directly has been found to date (Tamegai *et al.* 2007). However, possibility of such direct conversions has been postulated by Costa *et al.* (2006).

In support to the above, efforts were made to amplify *amoA* gene for ammonia oxidation and *nirS* and *nirK* gene for nitrite reduction. In several of the isolates these genes could be amplified even though there were band shifts. Failure to amplify these genes in other isolates which expressed the nitrifying and denitrifying potency phenotypically does not suggest their absence as much more standardization of the PCR reactions have to be carried out before coming to a conclusion. Homologues of *nirK* and *norB* have been fully sequenced in *N. europaea,* and partial *nirK* homologues have also been identified in several marine nitrifiers that are capable of nitrifier - denitrification. Regardless of the specific functions of *nirK* and *norB* in AOB, the presence of such sequences in AOB and the potential for different uses and controls relative to denitrifying bacteria complicates the interpretations of functional gene diversity in mixed environmental samples. The gene *nirS* has not been identified in nitrifying bacteria so far, but *nirK* has been reported in both nitrifying and denitrifying bacteria (Casciotti and Ward, 2005).

Table 2.1: Activity during amplification of NBC

Consortia	TAN removal mg/L/day	NO_2^--N production (mg/Lday)	NO_3^--N production (mg/L)
AMONPCU	6.57 ± 0.68	0.79 ± 0.48	BDL
AMOPCU	6.78 ± 1.69	0.18 ± 0.11	BDL
NIONPCU	5.74 ± 1.04	-	BDL
NIOPCU	6.41 ± 1.43	-	BDL

BDL: Below detectable limit.

Table 2. 2. Colony and cellular morphology, Gram's reaction and heterotrophic growth of isolates resolved from AMONPCU-1

Culture No.	Colony morphology on phytagel plates.	Gram's reaction	Cellular morphology	Heterotrophic growth(+/-)
N1, N3, N4, N5, N6, N9, N10, N11, N12, N13, N14, N15, N16. (14)	Pink circular, erose, umbonate, actinomycetes like.	+	Short rods	+
N2, N17 (2)	Transparent, irregular, lobate, flat.	+	Rods	+
N7, N18 (2)	Transparent, irregular, lobate, flat.	-	Rods	+

Table 2.3. Colony and cellular morphology, Gram's reaction and heterotrophic growth of isolates resolved from AMOPCU-1

Culture No.	Colony morphology on phytagel plates.	Gram's reaction	Cellular morphology	Heterotrophic growth(+/-)
N19, N20, N24 (3)	Grey, circular, erose, umbonate, actinomycetes like.	+	Short rods	+
N21, N25, N32 (3)	Whitish, pinpoint, entire, flat.	+	Rods	+
N22, N23, N26, N27, N28, N29, N30, N31, N33 (9)	Whitish, irregular, lobate, flat.	-	Rods	+

Table 2.4. Colony and cellular morphology, Gram's reaction and heterotrophic growth of isolates resolved from NIONPCU-1

Culture No.	Colony morphology on phytagel plates.	Gram's reaction	Cellular morphology	Heterotrophic growth(+/-)
N34, N35, N36, N37, N43, N45, N67 (7)	Pink circular, erose, umbonate, actinomycetes like.	+	Short rods	+
N39, N40 (2)	Grey, circular, erose, umbonate, actinomycetes like.	+	Short rods	+
N42, N44, N73 (3)	White/transparent, irregular, lobate, flat.	-	Cocci	+
N38, N41, N79, N80, N81 (5)	Pink circular, erose, flat, actinomycetes like.	+	Short rods	+
N46, N47, N49, N51, N56, N57, N58, N61, N62, N63, N64, N66, N70, N71, N76, N77, N78 (17)	Pink circular, undulate, umbonate, actinomycetes like.	+	Short rods	+
N52 (1)	Whitish, pinpoint, entire, raised.	+	Cocci	+
N50 (1)	Whitish, irregular, lobate, flat.	+	Short rods	+
N53, N60, N72, N75 (4)	Whitish, irregular, lobate, flat.	-	Rods	+
N54, N59, N65 (3)	Whitish, irregular, lobate, flat.	+	Cocci	+
N55, N74 (2)	Whitish, irregular, lobate, flat.	-	Cocci	+
N68, N69 (2)	White creamy, pinpoint, entire, convex.	+	Cocci	+
N48 (1)	White/transparent, irregular, lobate, flat.	+	Cocci	+

Table 2.5. Colony and cellular morphology, Gram's reaction and heterotrophic growth of isolates resolved from NIOPCU-1

Culture No.	Colony morphology on phytagel plates.	Gram's reaction	Cellular morphology	Heterotrophic growth(+/-)
N82	Pink, circular, undulate, umbonate, actinomycetes like.	+	Short rods	+
N83, N85, N86, N87, N89, N90 (6)	White creamy, irregular, lobate, flat.	-	Rods	+
N84	White, irregular, lobate, flat.	+	Short rods	+
N91	Pink, circular, undulate, umbonate, actinomycetes like.	+	Cocci	+
N92	White, irregular, lobate, flat.	+	Short rods	+
N93, N94, N96 (3)	Pink, circular, crose, umbonate, actinomycetes like.	+	Short rods	+
N95	Whitc/ transparent, irregular, lobate, flat.	-	Rods	+

Fig. 2.1. Dendrogram of pure culture isolated from AMONPCU-1 consortia.

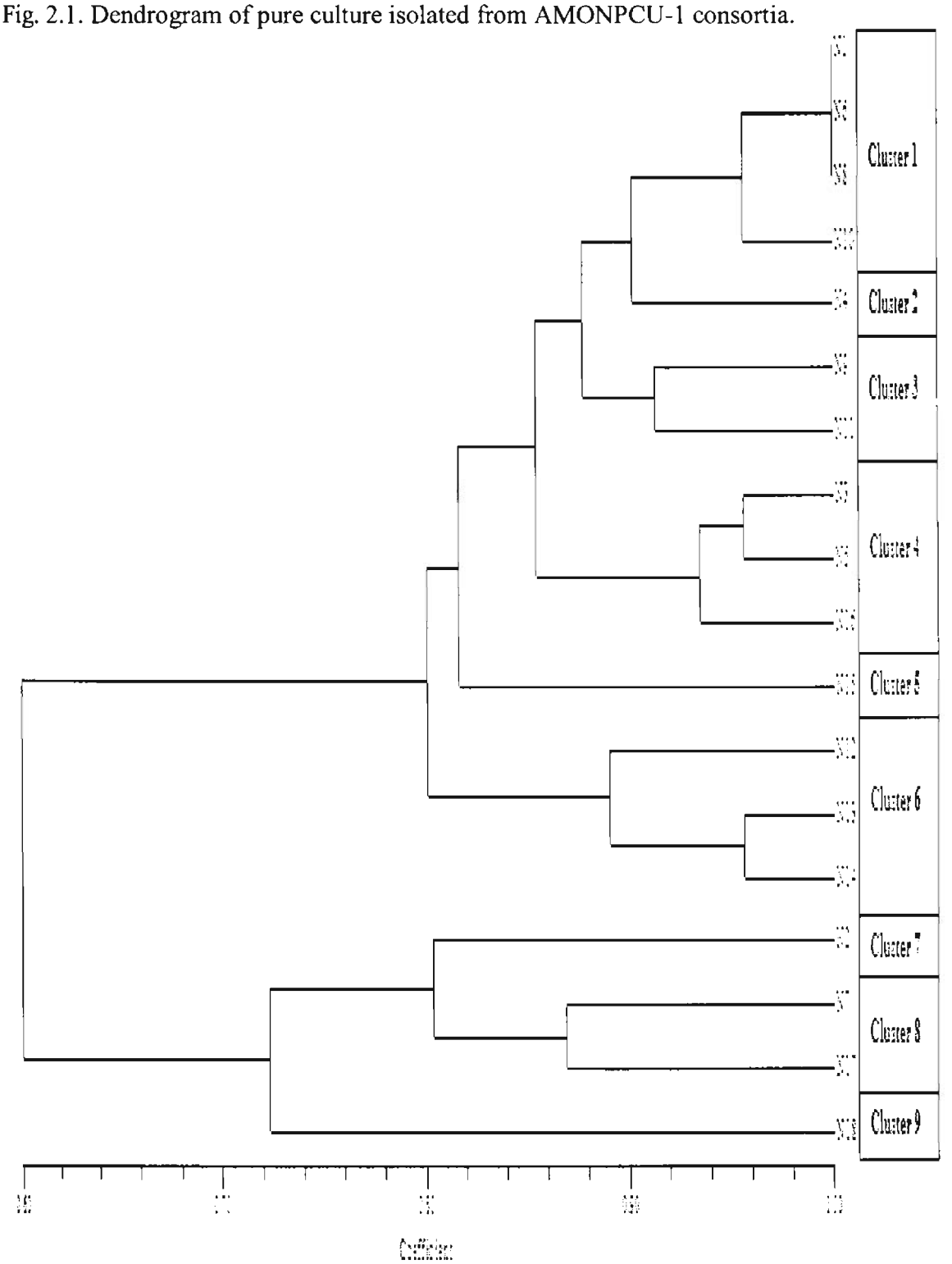


Fig. 2.2. Dendrogram of pure culture isolated from AMOPCU-1 consortia.

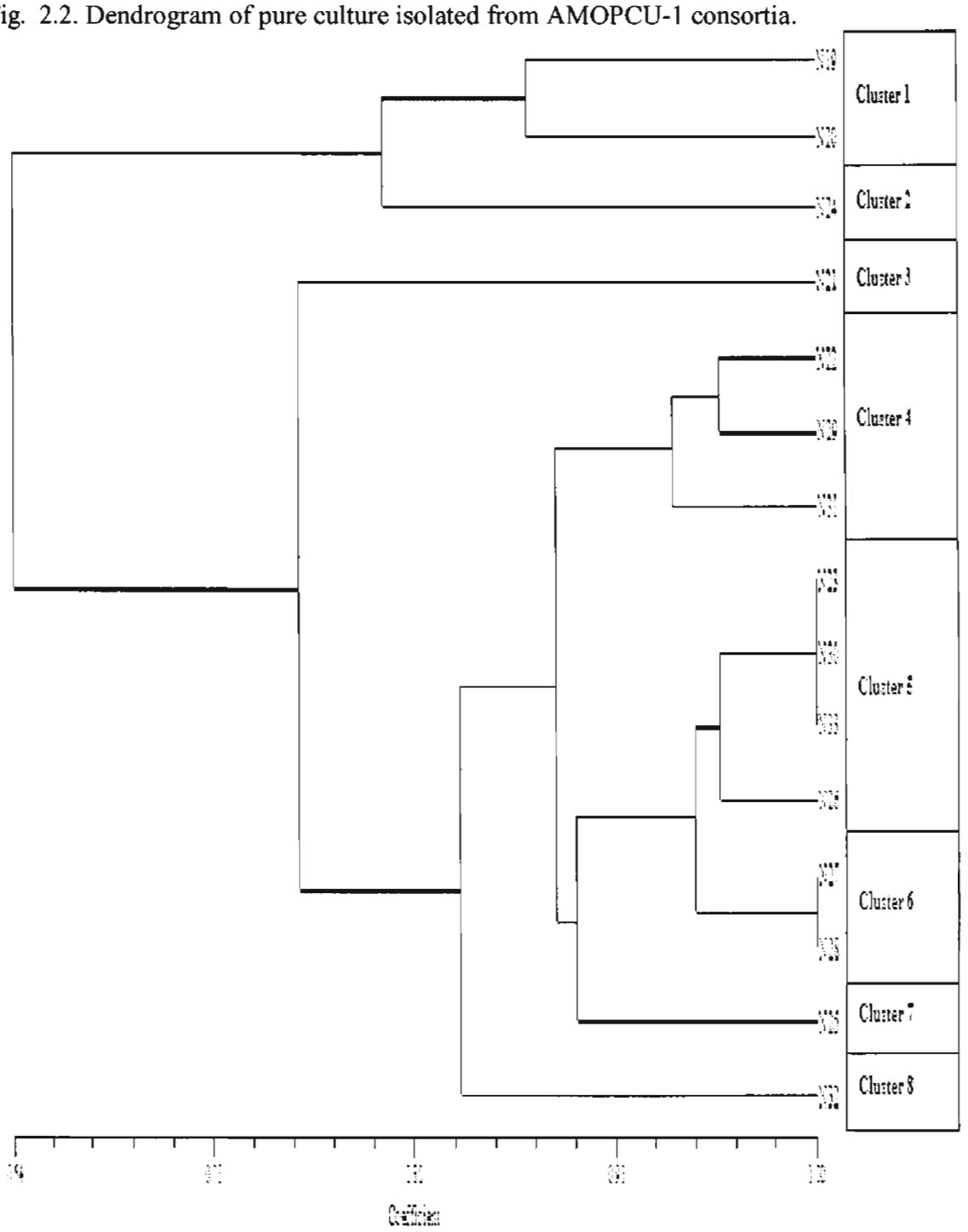


Fig. 2.3. Dendrogram of pure culture isolated from NIONPCU-1 consortia.

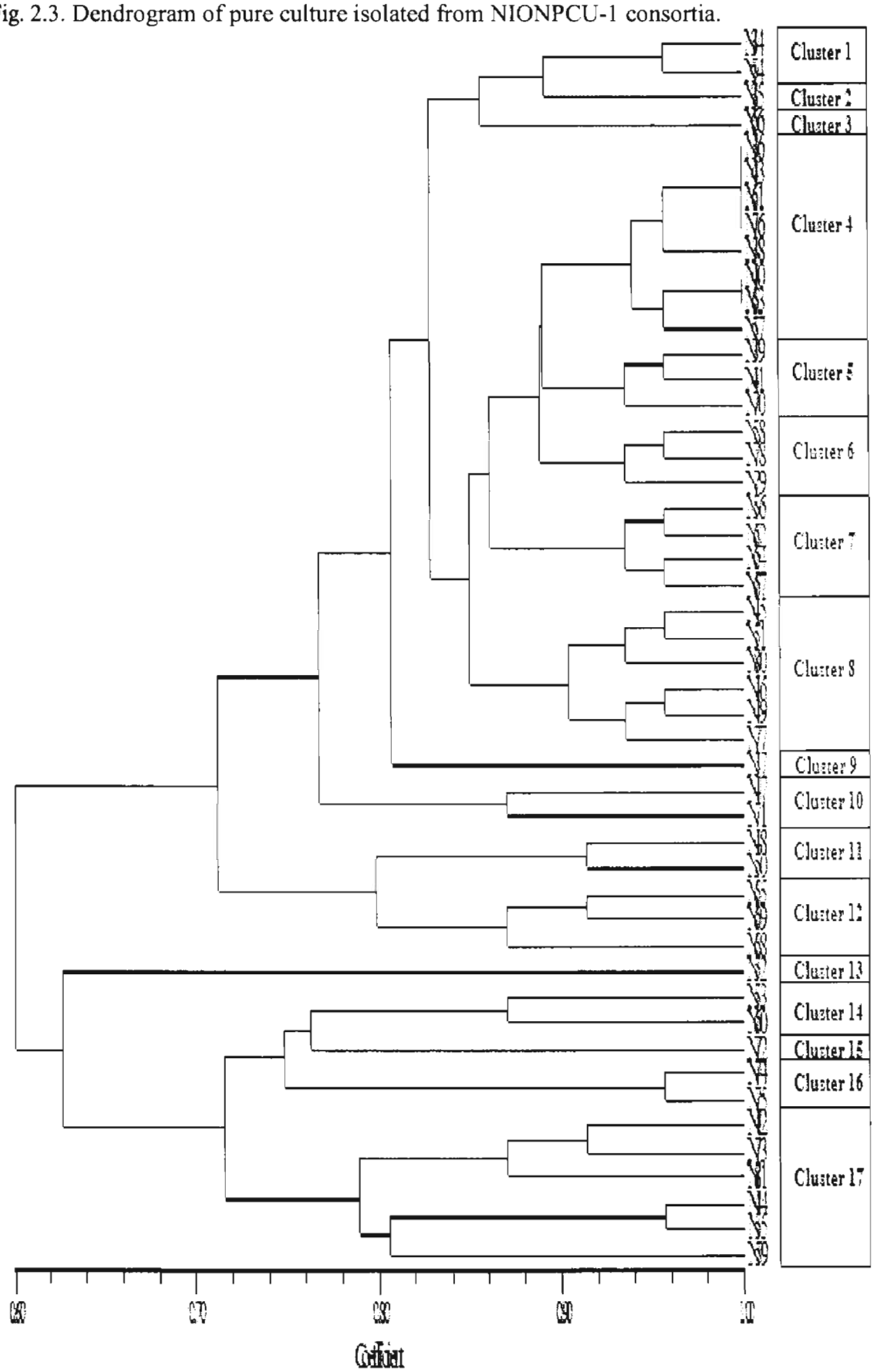


Fig. 2.4. Dendrogram of pure culture isolated from NIOPCU-1 consortia.

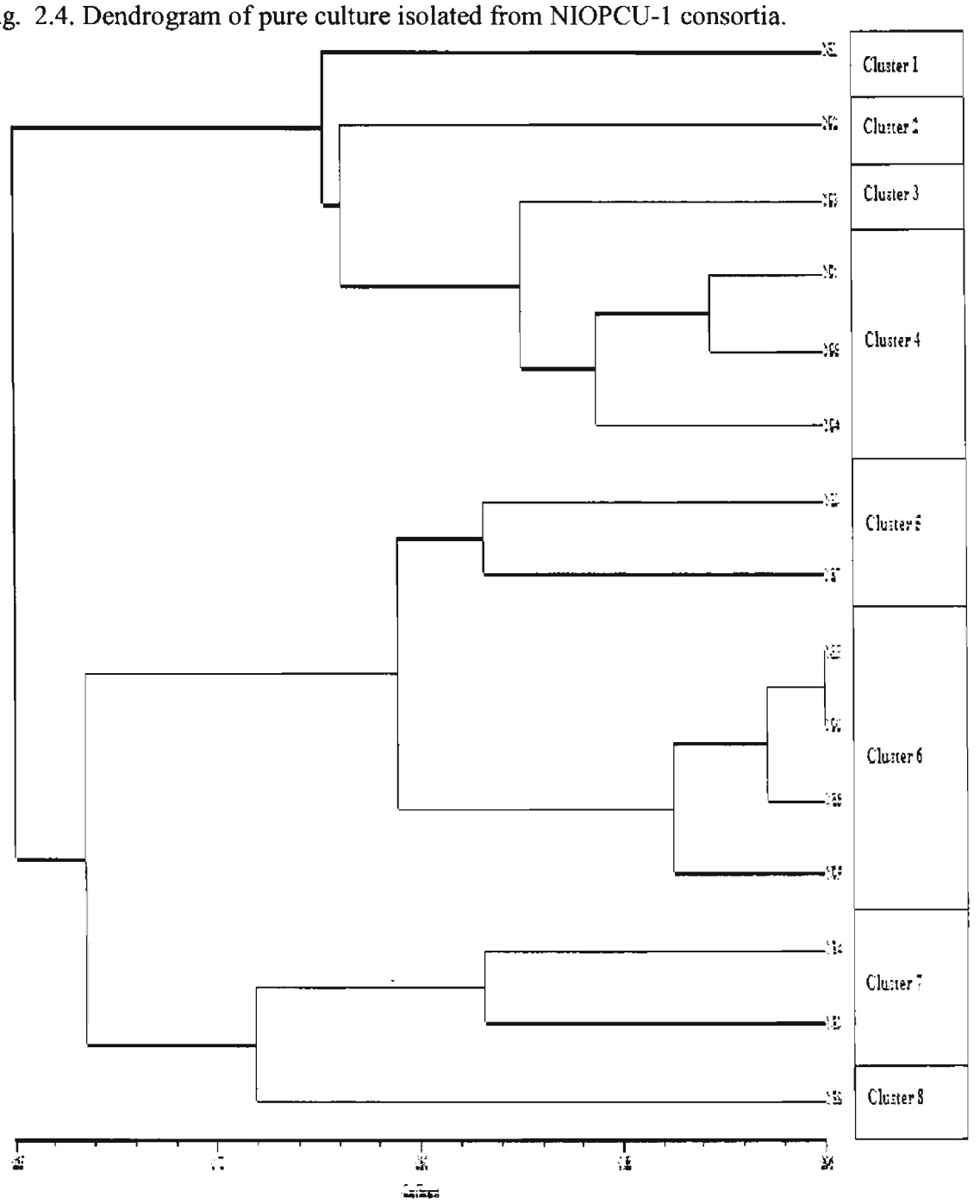


Table 2.6. Biochemical characterization of pure cultures (18 nos.) isolated from AMONPCU-1.

Reactions/attributes	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18
Gram's reaction	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0
Cellular morphology *	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Motility	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1
MOF reaction #	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Spore staining	0	0	0	0	0	0	NA	0	0	0	0	0	0	0	0	0	0	NA
Acid fast staining	0	0	0	0	0	0	NA	0	0	0	0	0	0	0	0	0	0	NA
Catalase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Kovac's oxidase	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Gelatinase	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1
Amylase	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1
DNAase	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	1	1
Lipase	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0
Cellulase	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
Chitinase	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0
Aesculin hydrolysis	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1
Phenyl alanine deaminase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gluconate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ONPG(β Galactosidase)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Indole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methyl red	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	1	1	0
Voges proskauer	0	0	1	1	1	0	0	0	1	0	1	1	1	0	0	1	1	1
Nitrate reduction	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0
Nitrite reduction	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	9
Citrate utilization	1	1	1	1	0	1	0	1	1	1	1	0	1	1	0	0	0	0

1: positive, 0: negative; * 1: rods and 0: cocci; # 1: oxidative, 0: fermentative and 9: alkaline/ no reaction.

Table 2.7. Biochemical characterization of pure cultures (15 Nos.) isolated from AMOPCU-1.

Reactions/attributes	N19	N20	N21	N22	N23	N24	N25	N26	N27	N28	N29	N30	N31	N32	N33
Gram's reaction	1	1	1	0	0	1	1	0	0	0	0	0	0	1	0
Cellular morphology *	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Motility	0	0	0	1	1	0	1	1	1	1	1	1	0	0	1
MOF reaction #	1	1	9	1	1	1	1	1	1	1	1	1	1	1	1
Spore staining	0	0	0	NA	NA	0	0	NA	NA	NA	NA	NA	NA	0	NA
Acid fast staining	0	0	0	NA	NA	0	0	NA	NA	NA	NA	NA	NA	0	NA
Catalase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Kovac's oxidase	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1
Gelatinase	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0
Amylase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
DNAase	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Lipase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cellulase	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Chitinase	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
Aesculin hydrolysis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenyl alanine deaminase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gluconate	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0
ONPG(β Galactosidase)	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Indole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methyl red	1	0	1	0	1	1	1	1	1	1	0	1	0	1	1
Voges proskauer	1	0	1	0	1	1	1	1	1	1	1	1	0	1	1
Nitrate reduction	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Nitrite reduction	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
Citrate utilization	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1

1: positive, 0: negative; * 1: rods and 0: cocci; # 1: oxidative, 0: fermentative and 9: alkaline/ no reaction.

Table 2.8: Biochemical characterization of pure cultures (48 Nos.) isolated from NIONPCU-1.

Reactions/attributes	N34	N35	N36	N37	N38	N39	N40	N41	N42	N43	N44	N45	N46	N47	N48	N49	N50	N51	N52	N53	N54	N55	N56	N57	N58	N59	N60	N61	N62	N63	N64	N65	N66	N67	N68	N69	N70	N71	N72	N73	N74	N75	N76	N77	N78	N79	N80	N81
Gram's reaction	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1
Cellular morphology *	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	0	1	0	0	1	1	1	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0
Motility	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0
MOF reaction #	1	1	1	1	1	1	1	1	9	1	9	1	1	9	1	1	1	1	1	1	1	9	1	1	1	9	1	1	1	1	1	1	1	1	1	1	1	9	1	9	9	9	1	1	1	1	1	9
Spore staining	0	0	0	0	0	0	0	0	NA	0	NA	0	0	0	NA	0	0	0	NA	NA	NA	NA	0	0	0	NA	NA	0	0	0	0	NA	0	0	NA	NA	0	0	NA	NA	NA	NA	0	0	0	0	0	NA
Acid fast staining	0	0	0	0	0	0	0	0	NA	0	NA	0	0	0	NA	0	0	0	NA	NA	NA	NA	0	0	0	NA	NA	0	0	0	0	NA	0	0	NA	NA	0	0	NA	NA	NA	NA	0	0	0	0	0	NA
Catalase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Kovac's oxidase	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
Gelatinase	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0
Amylase	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	0
DNAase	1	1	1	0	0	1	1	1	0	1	0	0	1	1	1	1	0	0	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	0	0
Lipase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1
Cellulase	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0
Chitinase	1	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1	0	1	0	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	0	1	1	1	1	1	0
Aesculin hydrolysis	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Phenyl alanine deaminase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
Gluconate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ONPG(β Galactosidase)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Indole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methyl red	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0
Voges proskauer	1	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	1	0	0	1	1	1	1	1	1	1	0
Nitrate reduction	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	0	1	0	0	1	1	1	0	0	1	1	1	1	0	0	1	0	0	1	1	0	0	0	0	1	1	1	1	1	0
Nitrite reduction	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Citrate utilization	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1	0	1

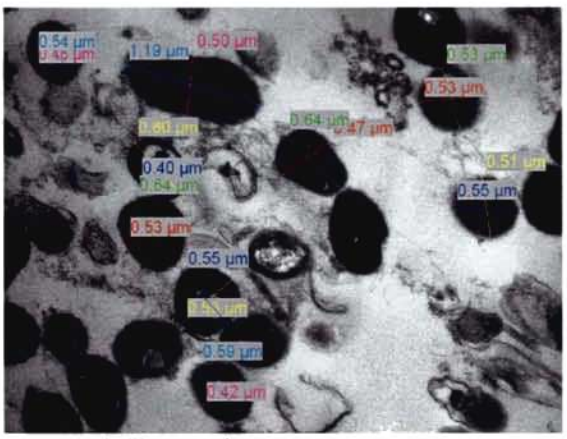
1: positive, 0: negative; * 1: rods and 0: cocci; # 1: oxidative, 0: fermentative and 9: alkaline/ no reaction.

Table 2.9. Biochemical characterization of pure cultures (15 Nos.) isolated from NIOPCU-1.

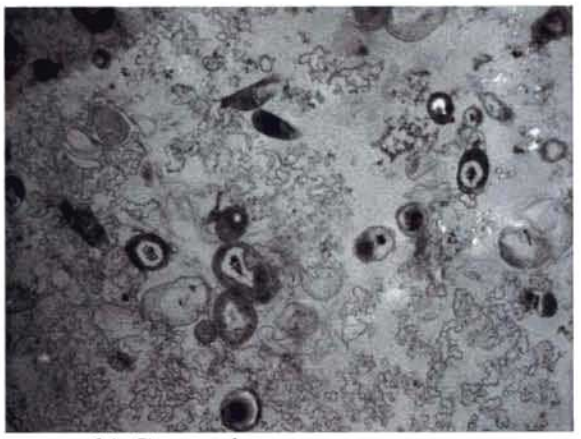
Reactions/attributes	N82	N83	N84	N85	N86	N87	N88	N89	N90	N91	N92	N93	N94	N95	N96
Gram's reaction	1	0	1	0	0	0	1	0	0	1	1	1	1	0	1
Cellular morphology *	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1
Motility	0	0	0	1	1	1	0	0	1	0	0	0	0	1	0
MOF reaction #	1	1	9	1	1	1	9	9	1	1	1	1	1	1	1
Spore staining	0	NA	0	NA	NA	NA	NA	NA	NA	NA	0	0	0	NA	0
Acid fast staining	0	NA	0	NA	NA	NA	NA	NA	NA	NA	0	0	0	NA	0
Catalase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Kovac's oxidase	0	1	0	1	1	1	0	1	1	0	1	0	0	1	0
Gelatinase	1	0	1	0	0	0	0	1	0	1	1	1	1	0	1
Amylase	1	0	0	1	1	0	0	0	1	1	1	1	1	1	1
DNAase	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Lipase	1	0	1	1	1	0	0	1	1	1	1	1	1	1	1
Cellulase	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
Chitinase	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aesculin hydrolysis	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
Phenyl alanine deaminase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Gluconate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ONPG(β Galactosidase)	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Indole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methyl red	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Voges proskauer	1	0	0	1	0	1	0	0	1	0	1	1	0	0	0
Nitrate reduction	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0
Nitrite reduction	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1
Citrate utilization	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1

1: positive, 0: negative; * 1: rods and 0: cocci; # 1: oxidative, 0: fermentative and 9: alkaline/ no reaction.

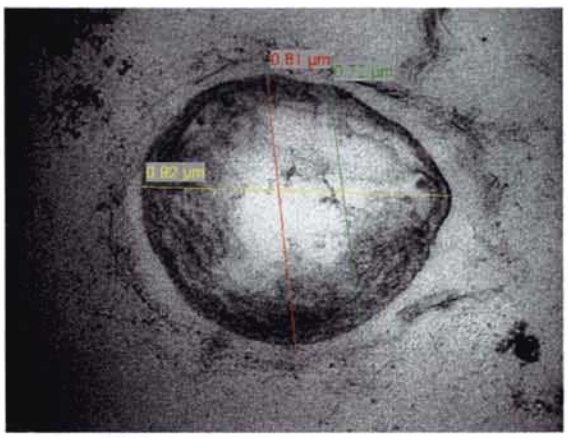
Fig. 2.5. TEM of the consortium AMONPCU-1.



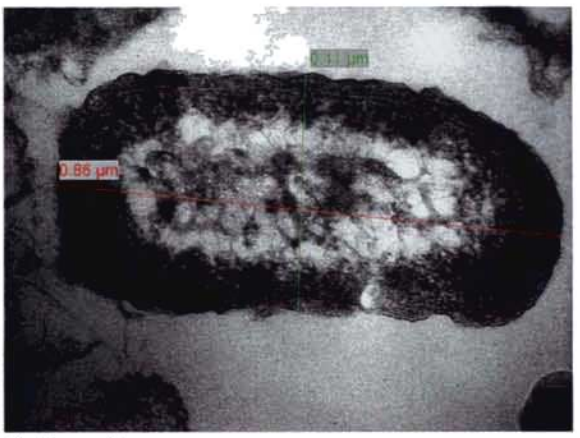
a) Consortium



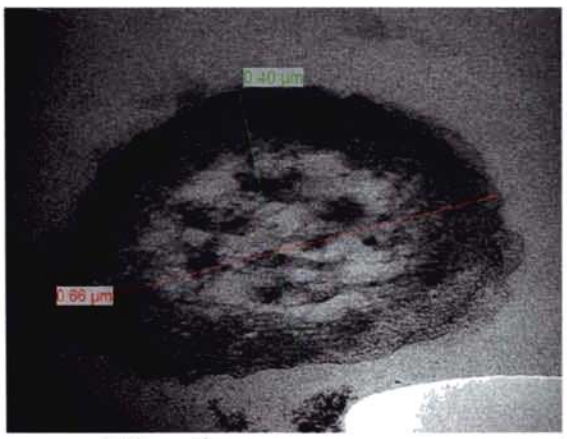
b) Consortium



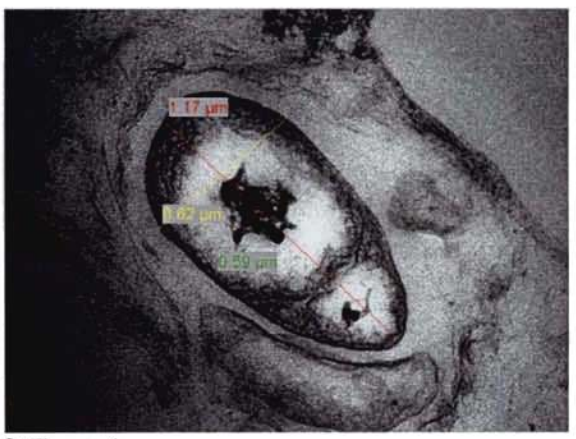
c) Type-1



d) Type-2

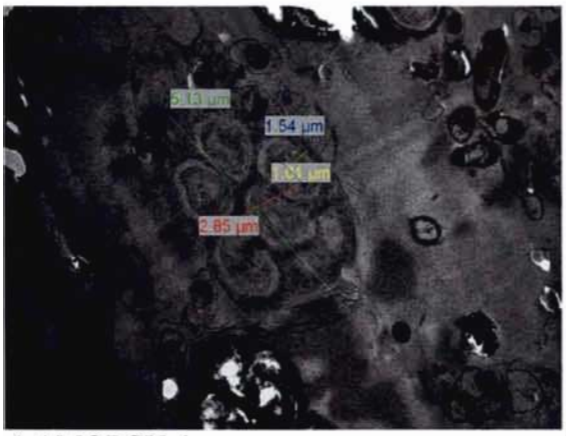


e) Type-3

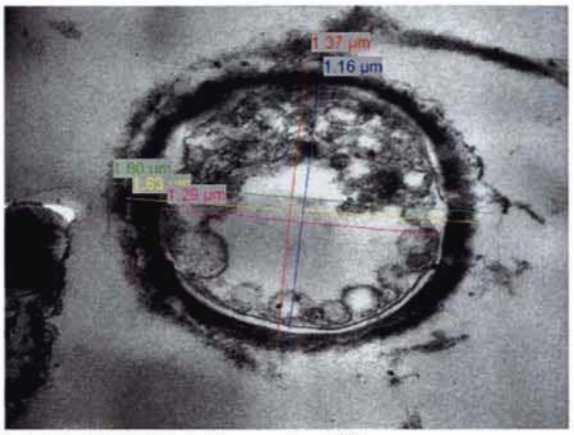


f) Type-4

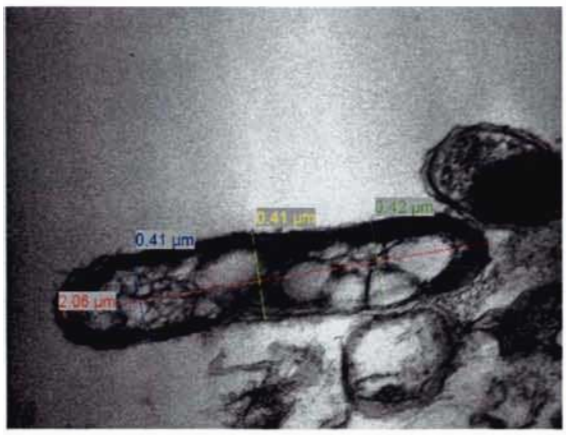
Fig. 2.6. TEM of the consortium AMOPCU-1



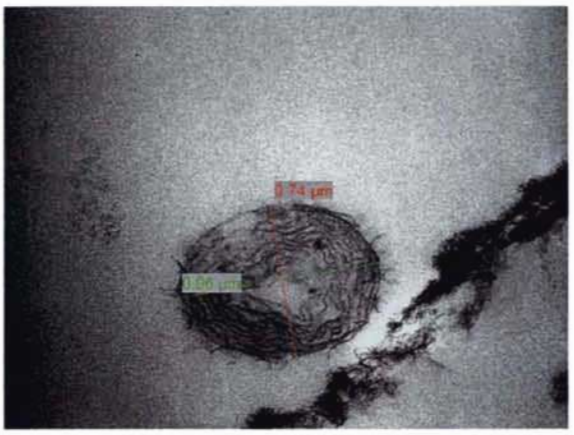
a) AMOPCU-1



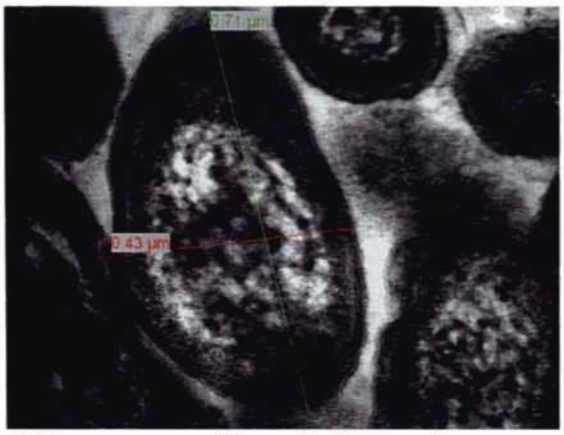
b) AMOPCU-1 (type-1 cell)



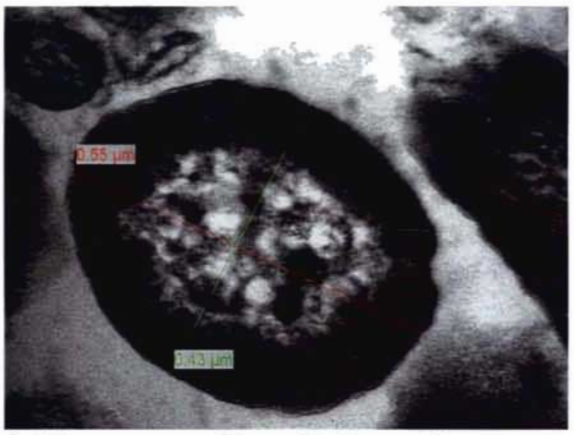
c) AMOPCU-1 (type-2 cell)



d) AMOPCU-1 (type-3 cell)

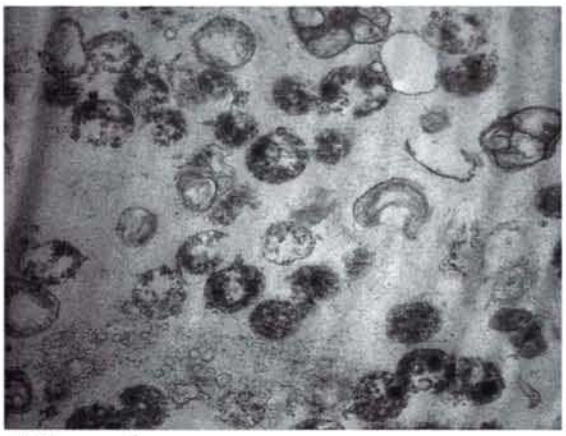


e) *Nitrosomonas* like cell: Pure culture

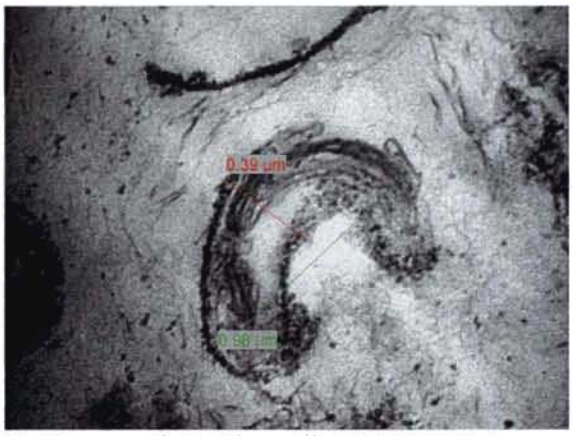


f) *Nitrosomonas* like cell: Pure culture

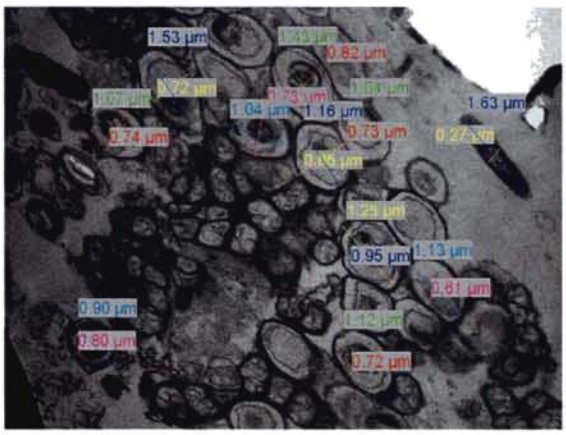
Fig. 2.7. TEM of the consortium NIONPCU-1



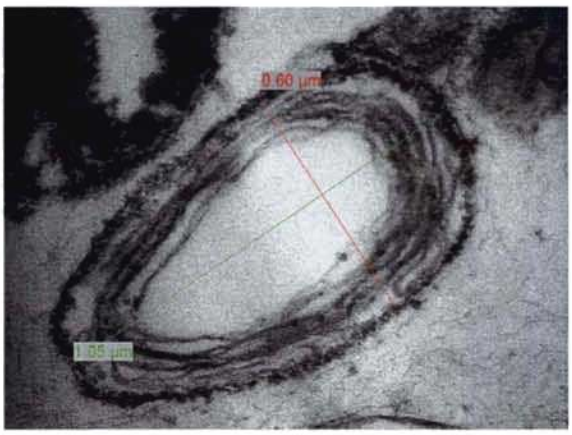
a) Consortium



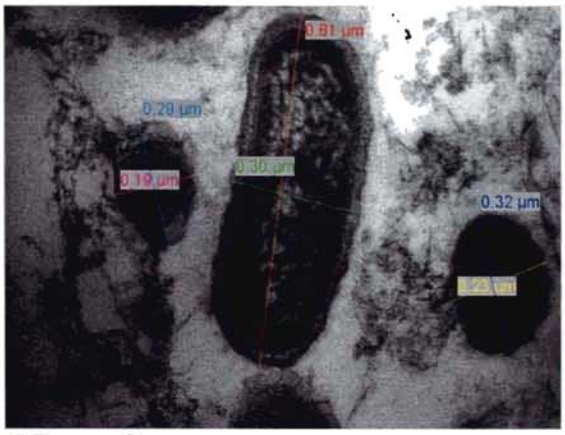
b) *Nitrosovibrio* like cell: NIONPCU-1



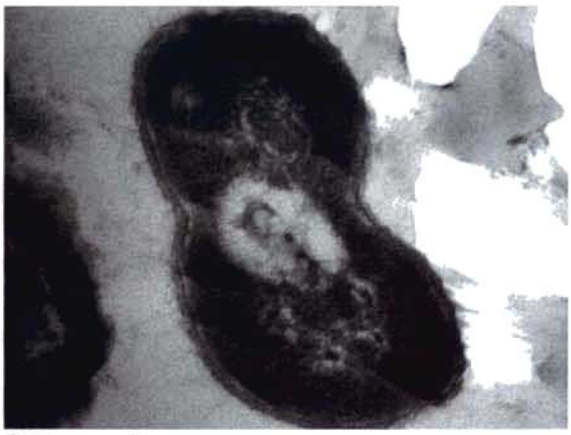
c) Zoogloea NIONPCU-1



d) NIONPCU-1

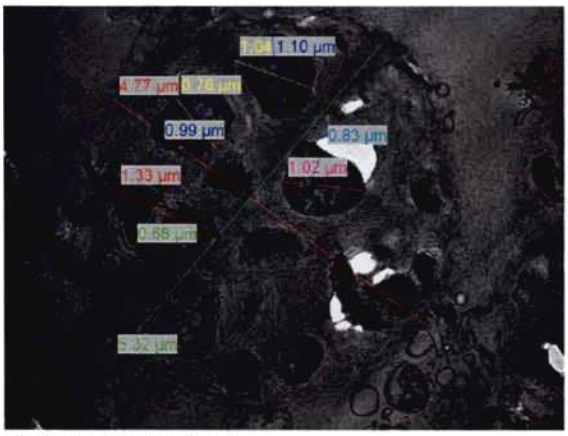


e) Pure culture

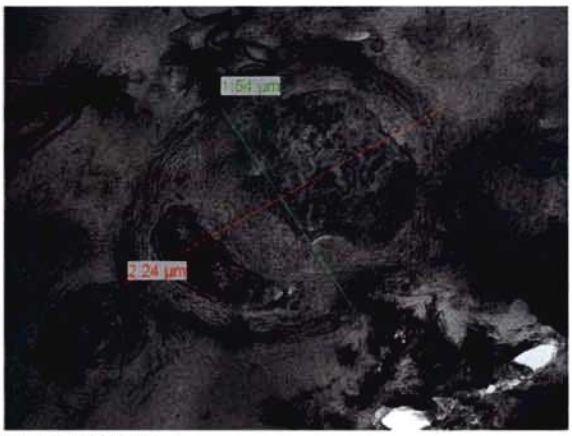


f) Pure culture

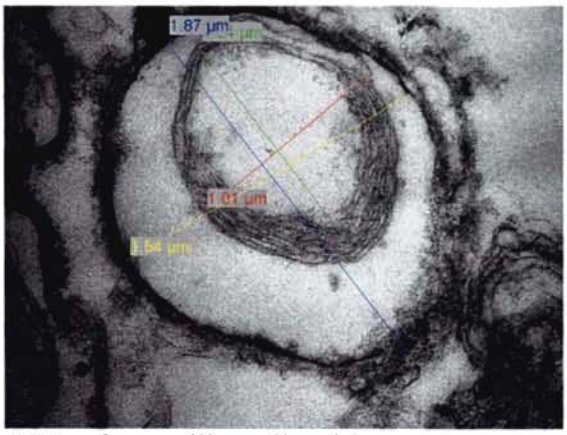
Fig. 2.8. TEM of the consortium NIOPCU-1



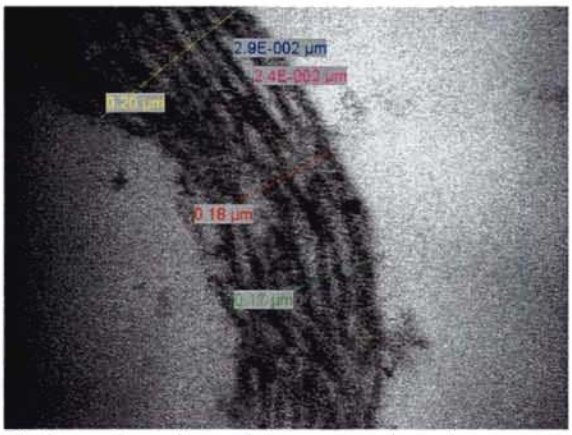
a) Cyst NIOPCU-1



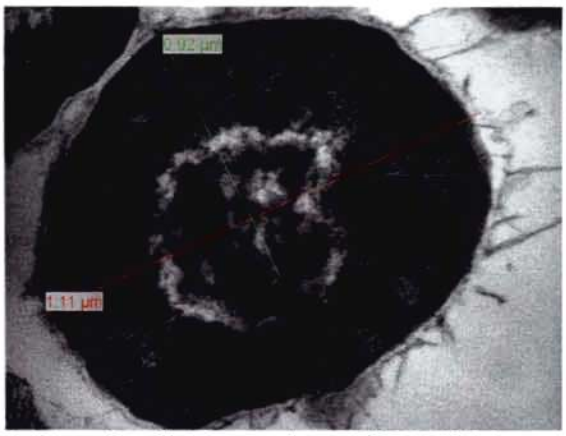
b) NIOPCU-1



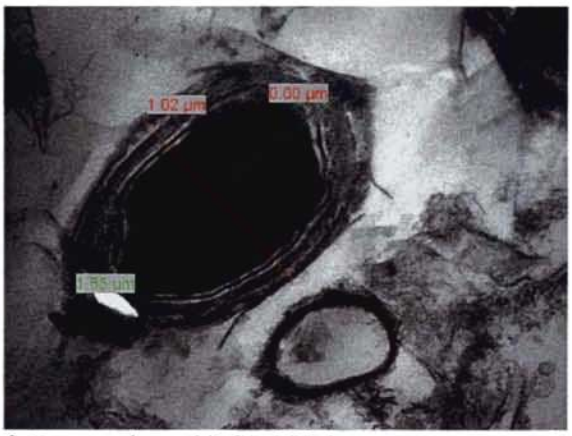
c) *Nitrobacter* like cells with intracytoplasmic membranes.



d) Cytoplasmic and intracytoplasmic membranes of *Nitrobacter* like cells



e) Type-2 cell: Pure culture NIOPCU-1



f) Pure culture NIOPCU-1

Table 2.10. Nitrification potency of the pure cultures isolated from ammonia oxidizing consortia (AMONPCU-1 and AMOPCU-1).

Culture No.	Nitrifying potency. (Aerated)	Total TAN consum ption. (mg/L)	Total nitrite build up (mg/L)	Day on which max. nitrite producti on detected.	Total nitrate build up (mg/L)	Day on which max. nitrate producti on detected.	Nitrifying potency. With out aeration.
N1	+	10.0	5.7	10	2.2	9	-
N2	+	5.0	4.6	1	BDL	-	-
N3	+	10.0	3.7	10	3.2	9	-
N4	+	4.0	4.5	1	BDL	-	-
N5	+	4.5	4.5	1	BDL	-	-
N6	+	9.3	BDL	-	3.1	9	-
N7	+	15.2	0.8	10	2.8	9	-
N8	+	14.7	3.4	10	1.8	9	-
N9	+	13.4	BDL	-	6.0	9	-
N10	+	6.9	2.1	10	5.0	9	-
N11	+	10.0	1.5	10	1.9	9	-
N12	+	13.5	2.2	10	1.0	6	-
N13	+	5.2	BDL	-	4.0	1	-
N14	+	5.0	BDL	-	1.0	6	-
N15	+	8.7	BDL	-	8.1	3	-
N16	+	6.0	BDL	-	1.3	6	-
N17	+	9.0	BDL	-	2.3	6	-
N18	+	9.1	BDL	-	1.0	7	-
N19	+	7.5	BDL	-	5.9	2	-
N20	+	7.3	1.2	5	1.9	13	-
N21	+	10.2	BDL	-	1.7	6	-
N22	+	9.8	BDL	-	3.2	4	-
N23	+	3.8	BDL	-	1.9	1	-
N24	+	9.2	BDL	-	6.0	4	-
N25	+	4.2	BDL	-	3.2	1	-
N26	+	9.2	BDL	-	0.8	4	-
N27	+	2.0	BDL	-	1.1	1	-
N28	+	6.3	BDL	-	2.4	1	-
N29	+	9.3	BDL	-	2.2	1	-
N30	+	3.4	BDL	-	2.9	1	-
N31	+	9.4	BDL	-	1.3	1	-
N32	+	9.2	BDL	-	1.9	4	-
N33	+	2.3	BDL	-	2.1	1	-

Cultures N1 - N18 (18Nos) from AMONPCU-1, N19 – N33 (15Nos) from AMOPCU-1
BDL = Below detectable limit.

Table 2.11. Nitrifying potency of pure cultures isolated from nitrite oxidizing consortia (NIONPCU-1 and NIOPCU-1).

Culture No	Nitrifying potency	Total nitrite consumption (mg/L)	Total nitrate build up (mg/L)	Day on which max. product detected	Nitrifying potency, with out aeration.
N34	+	2.5	2.4	4	-
N35	+	3.2	6.2	3	-
N36	+	3.7	3.9	4	-
N37	+	1.9	7.1	3	-
N38	+	4.3	4.3	5	-
N39	+	2.7	1.1	4	-
N40	+	10.1	9.8	4	-
N41	+	6.8	1.6	6	-
N42	+	8.0	6.8	4	-
N43	+	5.2	1.2	2	-
N44	+	6.3	1.0	5	-
N45	+	3.2	16.0	2	-
N46	+	9.3	0.7	7	-
N47	+	8.7	8.2	5	-
N48	+	10.2	0.3	2	-
N49	+	9.0	0.8	11	-
N50	+	9.8	BDL	-	-
N51	+	8.4	1.0	7	-
N52	+	6.3	2.0	5	-
N53	+	9.7	BDL	-	-
N54	+	8.8	1.2	6	-
N55	+	4.5	12.1	2	-
N56	+	6.4	2.6	5	-
N57	+	2.5	2.4	2	-
N58	+	21.0	47.8	12	-
N59	+	9.9	2.1	1	-
N60	+	10.1	2.1	1	-
N61	+	7.5	7.5	1	-
N62	+	9.5	BDL	-	-
N63	+	10.2	BDL	-	-
N64	+	3.1	2.1	4	-
N65	+	9.7	0.6	4	-
N66	+	7.5	0.7	11	-
N67	+	10.0	0.9	11	-
N68	+	8.9	4.0	5	-
N69	+	5.2	BDL	-	-
N70	+	4.8	4.7	1	-
N71	+	4.3	4.8	1	-
N72	+	3.2	1.2	1	-
N73	+	10.0	0.6	4	-
N74	+	5.2	4.9	3	-
N75	+	8.4	1.8	8	-

Table 2.11. Continuing

Culture No	Nitrifying potency	Total nitrite consumption (mg/L)	Total nitrate build up (mg/L)	Day on which max. product detected	Nitrifying potency, with out aeration.
N76	+	4.1	1.0	1	-
N77	+	7.1	4.5	2	-
N78	+	5.1	BDL	-	-
N79	+	5.4	1.0	3	-
N80	+	6.0	7.6	3	-
N81	+	4.4	2.2	7	-
N82	+	10.3	1.3	9	-
N83	+	10.0	4.3	4	-
N84	+	9.8	1.0	4	-
N85	+	8.3	1.7	4	-
N86	+	4.8	3.6	3	-
N87	+	4.1	1.7	3	-
N88	+	9.7	2.2	9	-
N89	+	4.5	3.3	2	-
N90	+	8.8	5.8	4	-
N91	+	4.5	3.6	5	-
N92	+	6.4	2.5	10	-
N93	+	6.3	1.6	3	-
N94	+	4.7	3.5	4	-
N95	+	6.4	2.8	2	-
N96	+	10.3	9.3	4	-

N34 - N81 (48Nos) from NIONPCU-1 and N82 - N96 (15Nos) from NIOPCU-1.
BDL: Below detectable limit.

Table 2.12. Denitrifying potency of the pure cultures isolated from all the four consortia

# Initial nitrate concentration in the medium 140ppm @ Initial nitrite concentration in the medium 231ppm			
Culture No.	Total nitrite production after 5 days (mg/L)	Total nitrate concentration after 5 days # (mg/L)	Nitrite concentration after 2 days @ (mg/L)
N1	3.99	BDL	122.1
N2	0.71	2.98	178.2
N3	1.60	BDL	165.0
N4	1.12	0.41	135.3
N5	3.92	BDL	128.7
N6	3.99	BDL	171.6
N7	0.96	BDL	155.1
N8	1.84	BDL	181.5
N9	3.79	BDL	174.9
N10	0.67	0.02	132.0
N11	32.07	BDL	168.3
N12	34.17	BDL	141.9
N13	30.69	BDL	141.9
N14	28.03	BDL	155.1
N15	35.22	BDL	198.0
N16	37.29	BDL	145.2
N17	0.61	2.14	204.6
N18	0.11	0.91	135.3
N19	BDL	2.14	181.5
N20	BDL	1.54	227.7
N21	BDL	0.26	79.2
N22	BDL	BDL	184.8
N23	BDL	0.07	99.0
N24	BDL	1.52	125.4
N25	BDL	39.35	168.3
N26	BDL	0.10	171.6
N27	27.81	BDL	178.2
N28	3.81	33.03	145.2
N29	BDL	7.45	194.7
N30	BDL	BDL	174.9
N31	BDL	0.32	115.5
N32	BDL	BDL	198.0
N33	BDL	48.82	221.1
N34	0.47	1.27	141.9
N35	0.44	1.85	211.2
N36	7.21	BDL	224.4
N37	2.38	BDL	171.6
N38	5.91	BDL	151.8
N39	4.55	BDL	178.2
N40	9.29	BDL	158.4
N41	2.70	BDL	BDL
N42	0.27	0.14	42.9
N43	1.22	1.66	196.0

Table 2.12 continuing

Culture No.	Total nitrite production after 5 days (mg/L)	Total nitrate concentration after 5 days # (mg/L)	Nitrite concentration after 2 days @ (mg/L)
N44	0.35	BDL	66.0
N45	4.96	BDL	151.8
N46	3.99	BDL	178.2
N47	25.06	BDL	158.4
N48	0.58	BDL	39.6
N49	3.15	0.12	201.3
N50	0.52	BDL	52.8
N51	28.98	BDL	181.5
N52	0.19	BDL	204.6
N53	22.48	BDL	191.4
N54	0.68	1.39	198.0
N55	0.42	0.32	89.1
N56	2.26	BDL	194.7
N57	1.14	0.72	204.6
N58	1.08	1.95	191.4
N59	0.51	0.09	105.6
N60	0.54	BDL	49.5
N61	2.28	BDL	214.5
N62	8.28	BDL	207.9
N63	3.80	1.2	204.6
N64	4.00	BDL	158.4
N65	0.33	0.53	211.2
N66	0.34	1.49	181.5
N67	2.72	0.10	210.9
N68	0.42	BDL	155.1
N69	0.42	0.15	135.3
N70	3.12	0.05	141.9
N71	30.82	BDL	145.2
N72	0.40	BDL	36.3
N73	0.29	BDL	75.9
N74	0.23	0.27	188.1
N75	0.23	BDL	168.3
N76	1.89	1.30	161.7
N77	2.36	0.46	207.9
N78	0.96	1.08	184.8
N79	2.62	0.34	151.8
N80	40.10	BDL	138.6
N81	0.39	BDL	36.3
N82	BDL	2.75	181.5
N83	BDL	BDL	52.8
N84	BDL	BDL	66.0
N85	BDL	96.63	171.6
N86	56.07	BDL	168.3
N87	0.30	BDL	165.0
N88	0.27	BDL	46.2

Table 2.12 continuing

Culture No.	Total nitrite production after 5 days (mg/L)	Total nitrate concentration after 5 days # (mg/L)	Nitrite concentration after 2 days @ (mg/L)
N89	0.29	BDL	59.4
N90	23.82	11.03	165.0
N91	BDL	3.33	138.6
N92	65.48	BDL	155.1
N93	4.89	BDL	138.6
N94	BDL	4.51	181.5
N95	BDL	62.16	171.6
N96	BDL	19.03	151.8

Cultures N1 - N18 (18Nos) from AMONPCU-1, N19 – N33 (15Nos) from AMOPCU-1, N34 - N81 (48Nos) from NIONPCU-1 and N82 - N96 (15Nos) from NIOPCU-1.
BDL: Below detection limit

Table 2.13: Nitrification and denitrification potential of all isolates.

Culture No.	Denitrification	Nitrification: Products detected		
		NO_2	NO_3	NO_2 & NO_3
N1	+			+
N2	+	+		
N3	+			+
N4	+	+		
N5	+	+		
N6	+		+	
N7	+			+
N8	+			+
N9	+		+	
N10	+			+
N11	+			+
N12	+			+
N13	+		+	
N14	+		+	
N15	+		+	
N16	+		+	
N17	+		+	
N18	+		+	
N19	+		+	
N20	+			+
N21	+		+	
N22	+		+	
N23	+		+	
N24	+		+	
N25	+		+	
N26	+		+	
N27	+		+	
N28	+		+	
N29	+		+	
N30	+		+	
N31	+		+	
N32	+		+	
N33	+		+	
N34	+		+	
N35	+		+	
N36	+		+	
N37	+		+	
N38	+		+	
N39	+		+	
N40	+		+	
N41	+		+	
N42	+		+	
N43	+		+	
N44	+		+	
N45	+		+	
N46	+		+	
N47	+		+	
N48	+		+	
N49	+		+	
N50	+		+	

Table 2.13. Continuing

Culture No.	Denitrification	Nitrification: Products detected		
		NO_2	NO_3	NO_2 & NO_3
N51	+		+	
N52	+		+	
N53	+		+	
N54	+		+	
N55	+		+	
N56	+		+	
N57	+		+	
N58	+		+	
N59	+		+	
N60	+		+	
N61	+		+	
N62	+		+	
N63	+		+	
N64	+		+	
N65	+		+	
N66	+		+	
N67	+		+	
N68	+		+	
N69	+		+	
N70	+		+	
N71	+		+	
N72	+		+	
N73	+		+	
N74	+		+	
N75	+		+	
N76	+		+	
N77	+		+	
N78	+	*	*	*
N79	+		+	
N80	+		+	
N81	+		+	
N82	+		+	
N83	+		+	
N84	+		+	
N85	+		+	
N86	+		+	
N87	+		+	
N88	+		+	
N89	+		+	
N90	+		+	
N91	+		+	
N92	+		+	
N93	+		+	
N94	+		+	
N95	+		+	
N96	+		+	

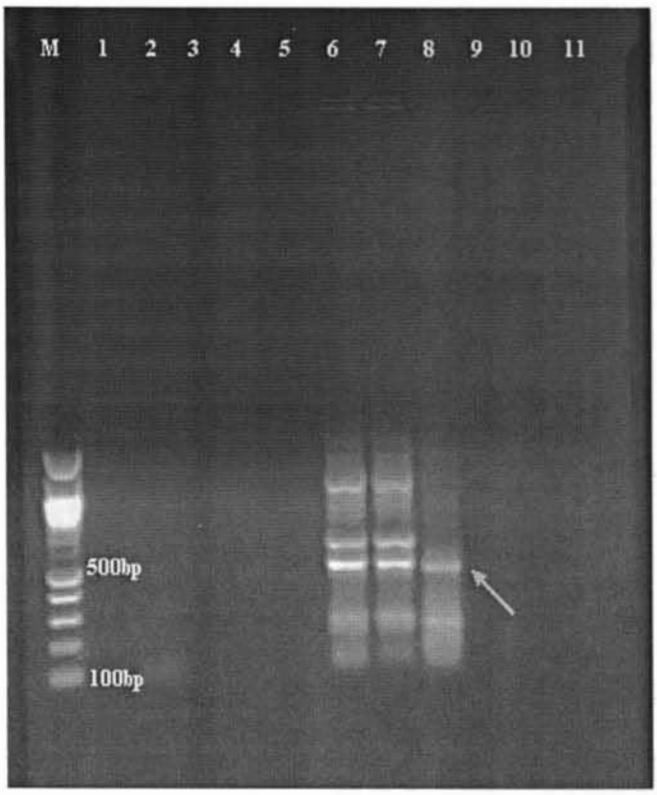
Cultures N1 - N18 (18Nos) from AMONPCU-1, N19 – N33 (15Nos) from AMOPCU-1, N34 - N81 (48Nos) from NIONPCU-1 and N82 - N96 (15Nos) from NIOPCU-1. * Products not detected.

Table 2.14: Isolates selected for PCR amplification of genes coding for AMO and NIR.

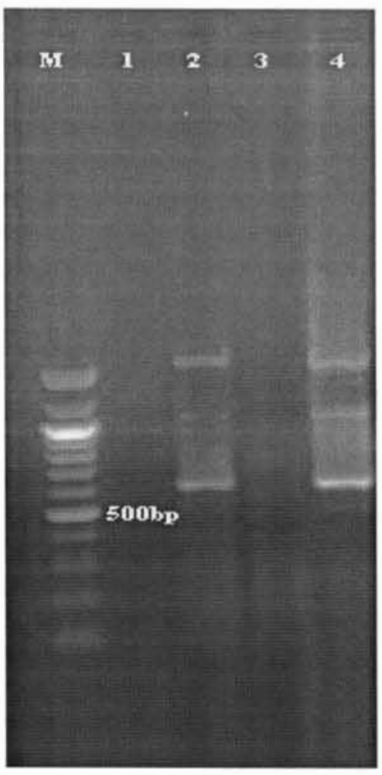
Culture No.	Source (consortia)	Gram's reaction	Nitrifying potency. product build up	Denitri-fying potency	*amoA*	*nirS*	*nirK*
N1	AMONPCU-1	+	NO_2, NO_3	+			
N2		+	NO_2	+			
N4		+	NO_2	+			
N5		+	NO_2	+			
N7		-	NO_2, NO_3	+			
N11		+	NO_2, NO_3	+		+/-	
N12		+	NO_2, NO_3	+			
N15		+	NO_3	+		+/-	
N18		-	NO_3	+		+	
N20	AMOPCU-1	+	NO_2, NO_3	+			
N21		+	NO_3	+			+/-
N24		+	NO_3	+		+	+/-
N25		+	NO_3	+		+	
N28		-	NO_3	+			
N29		-	NO_3	+	+	+	+/-
N30		-	NO_3	+	+	+	+/-
N32		+	NO_3	+	+/-	+/-	+
N82	NIOPCU-1	+	NO_3	+			+/-
N83		-	NO_3	+	+	+/-	
N86		-	NO_3	+			
N88		+	NO_3	+		+	+
N89		-	NO_3	+			
N91		+	NO_3	+			
N92		+	NO_3	+	+/-	+	
N93		+	NO_3	+			
N35	NIONPCU-1	+	NO_3	+			
N37		+	NO_3	+			
N39		+	NO_3	+		+	
N45		+	NO_3	+			
N47		+	NO_3	+		+/-	
N48		+	NO_3	+			
N52		+	NO_3	+			
N54		+	NO_3	+			
N55		-	NO_3	+			
N56		+	NO_3	+			
N58		+	NO_3	+			
N60		-	NO_3	+			
N61		+	NO_3	+			
N65		+	NO_3	+			
N66		+	NO_3	+			+/-
N72		-	NO_3	+		+	
N73		-	NO_3	+			
N74		-	NO_3	+			

+/- Band shift.

Fig. 2.9 Gel picture of *amoA* gene (491bp) amplified

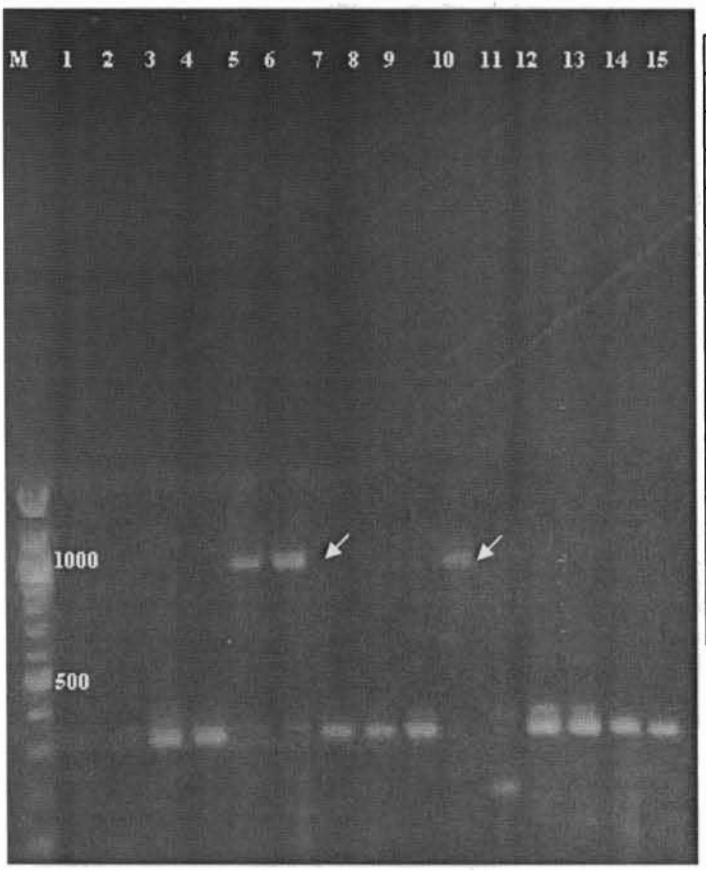


Lane number	Culture
1	N7
2	N55
3	N73
4	N74
5	N28
6	N29
7	N30
8	N83
9	N86
10	N89
11	+ control

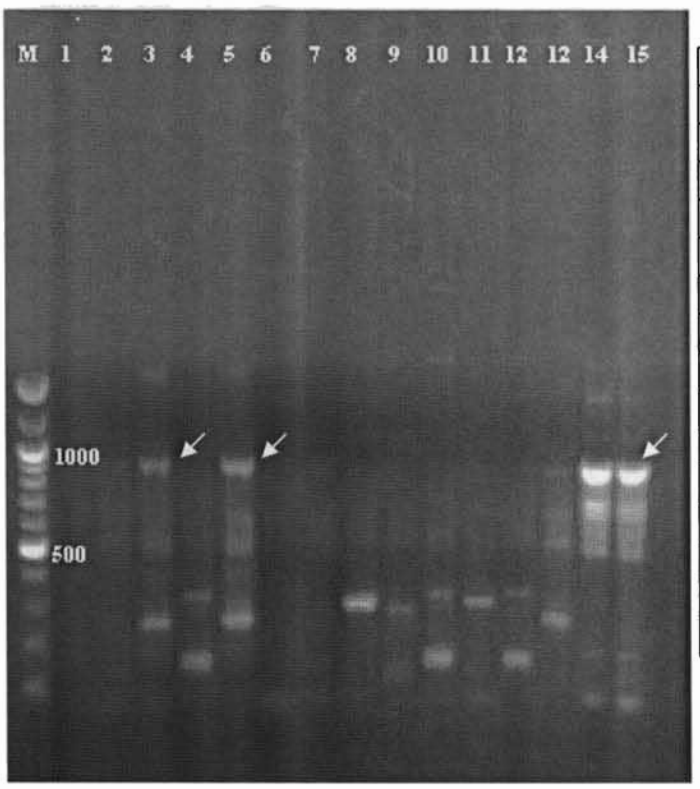


Lane number	Culture
1	-
2	N32
3	N88
4	N92

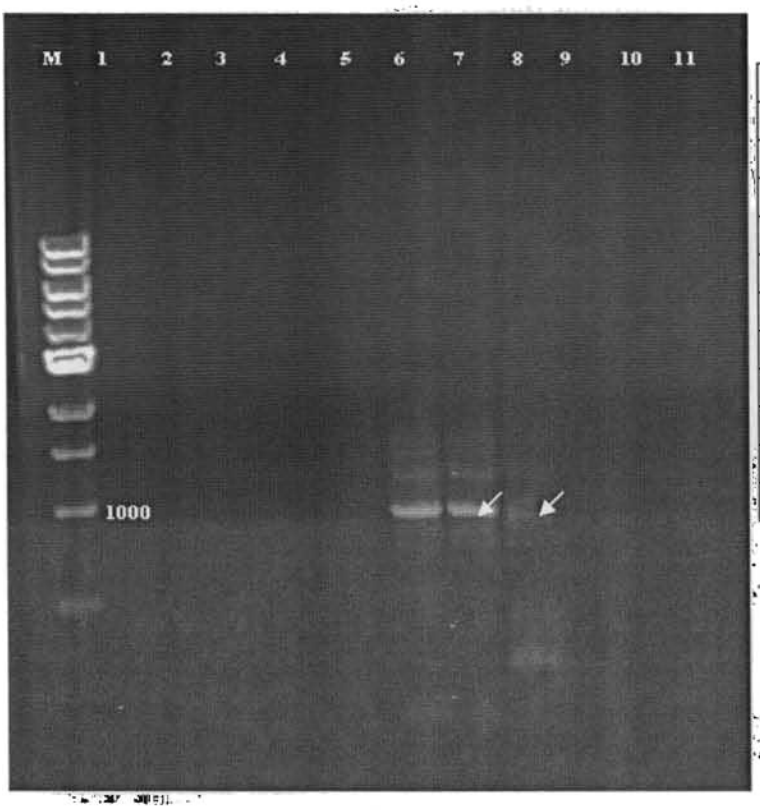
Fig. 2.10. Gel picture of *nirS* gene (890bp) amplified.



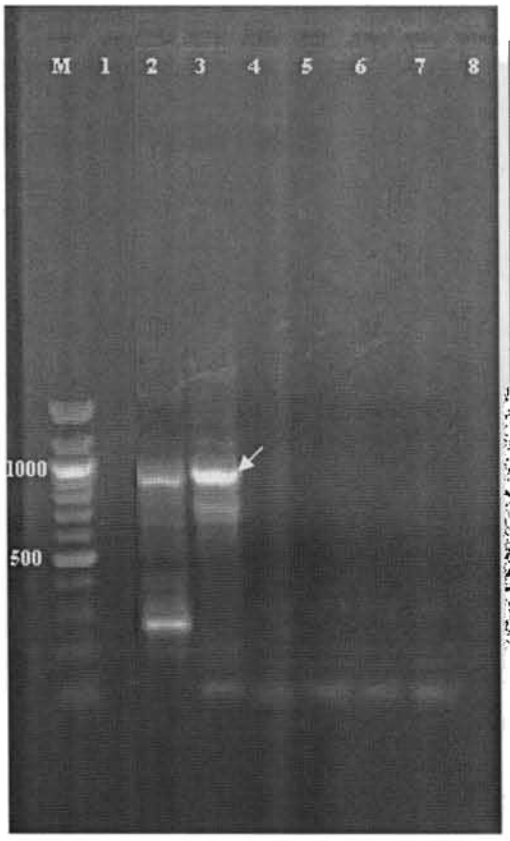
Lane	Culture
1	-
2	N1
3	N4
4	N5
5	N11
6	N15
7	N35
8	N37
9	N39
10	N47
11	N48
12	N56
13	N58
14	N66
15	N20



Lane	Culture
1	-
2	N7
3	N18
4	N60
5	N72
6	N2
7	N12
8	N45
9	N52
10	N54
11	N61
12	N65
13	N21
14	N25
15	N39

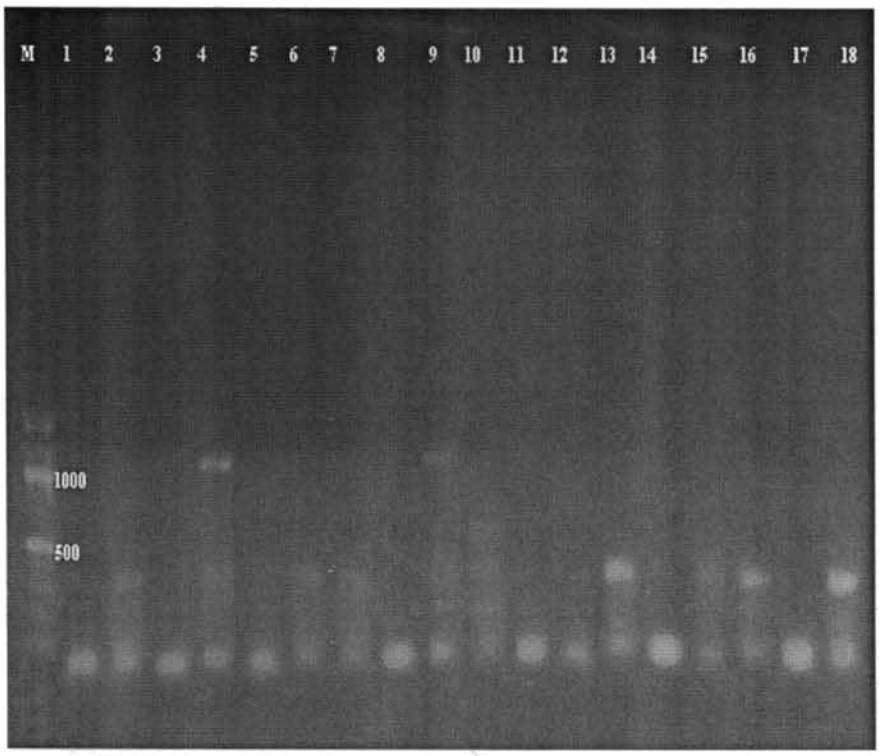


Lane	Culture
1	N7
2	N55
3	N73
4	N74
5	N28
6	N29
7	N30
8	N83
9	N86
10	N89
11	+ control

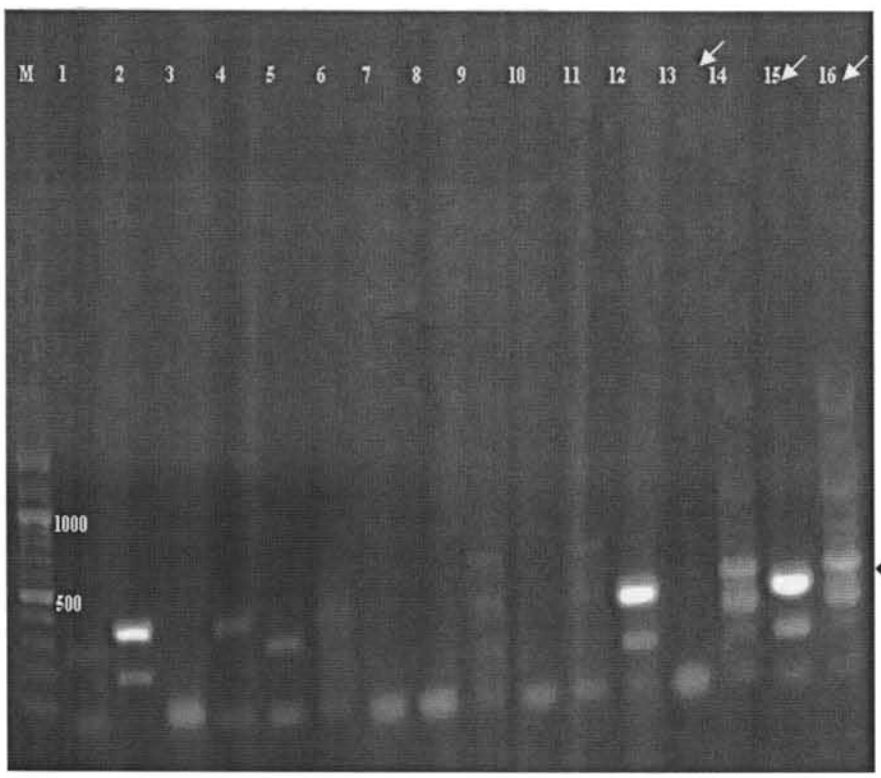


Lane	Culture
1	-
2	N88
3	N92
4	N24
5	N82
6	N91
7	N93
8	-

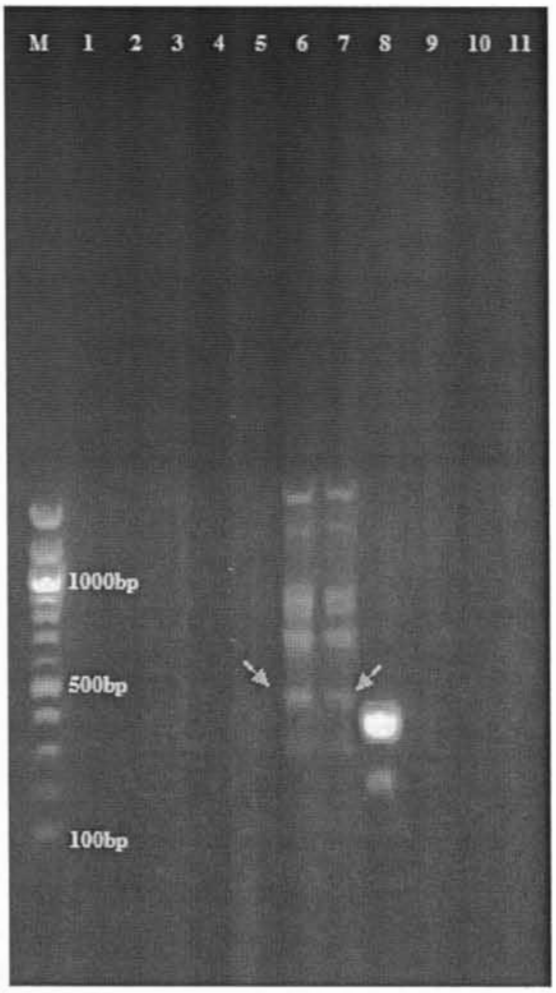
Fig. 2.11. Gel picture of *nirK* gene (514bp) amplified



Lane	Culture
1	N1
2	N4
3	N5
4	N11
5	N15
6	N35
7	N37
8	N39
9	N47
10	N48
11	N56
12	N58
13	N66
14	N20
15	N24
16	N82



Lane	Culture
1	N7
2	N18
3	N60
4	N72
5	N2
6	N12
7	N45
8	N52
9	N54
10	N61
11	N65
12	N21
13	N25
14	N32
15	N88
16	N92



Lane	Culture
1	N7
2	N55
3	N73
4	N74
5	N28
6	N29
7	N30
8	N83
9	N86
10	N89
11	+control

Chapter 3

Immobilization of nitrifying bacterial consortia on appropriate carrier material.

3.1. Introduction

Nitrogen is a major limiting nutrient in aquatic ecosystems and nitrogen loading often dictates phytoplankton density in aquaculture systems. Intensive versions of aquaculture depend entirely on nitrogenous inputs which ultimately contribute to nitrogenous waste loading. Fish expels various nitrogenous waste products through gill diffusion, gill cation exchange and as urine and feaces (Hagopian and Riley, 1998). One of the main problems, after dissolved oxygen in intensive shrimp culture is the accumulation of dissolved nitrogen, especially ammonia (sum of ionized and unionized species). Sometimes the term 'ammonia' is used to refer the total ammonia nitrogen (TAN) (Ebeling *et al.* 2006). High TAN accumulation in pond water can be deleterious to cultured organisms, optimum shrimp growth demands less than 0.1ppm unionized ammonia in culture ponds (1.33 to 1.53mg/L TAN at pH 8.0 and 28-30°C temperature – Shan and Obbard, 2001). High ammonia levels have often manifested as poor feed intake, retarded growth, poor survival and high susceptibility to diseases. Such situations are negotiated by huge pond water exchange; Deb (1998) reported 40% water exchange every few days to remove toxic waste metabolites from the culture system.

Increase in concentration of ammonia in receiving waters from shrimp farms have been observed by many researchers (Jones *et al.* 2001) and this has been correlated with nutrient enrichment leading to eutrophication in near by water bodies. It was reported that ammonia concentration increased in adjacent coastal creeks during shrimp culture period (Biao *et al.* 2004). Moreover, total pollution loading in the adjacent water bodies during aquaculture production cycle was strongly correlated with stocking density and estimated that the total nitrogen could be as high as 668 Kg/ha per cycle (Dierberg and Kiattisimkul, 1996).

An approximate value of ammonia-nitrogen generated per day in an aquaculture production system can be calculated based on the feeding rate (Ebeling *et al.* 2006).

$$P_{TAN} = F*PC*0.092$$

P_{TAN} is the production rate of total ammonia nitrogen (Kg/day), F is the feed rate (Kg/day) and PC is the protein concentration in feed (decimal value). The constant in the equation assumes that protein is 16% nitrogen, 80% nitrogen is assimilated by the organism, 80% assimilated nitrogen is excreted, and 90% of nitrogen excreted as TAN and 10% as urea. It also assumes that nitrogen in feaces and uneaten feed is promptly removed by

sedimentation or filtration and sludge removed from the system. This equation has been modified in the case of heterotrophic bacterial based zero exchange production system. In such a system all of the nitrogen excreted, both TAN and urea is available to the bacterial community since solid removal system and traditional fixed film biofilters are not used. Moreover 90% of assimilated nitrogen by marine shrimp is excreted as TAN and urea (Ebeling *et al.* 2006). The modified equation for marine shrimp is:

$$P_{TAN} = F*PC*0.144$$

3.1.1. TAN removal in aquaculture systems.

TAN can be removed from culture pond water by enhancing natural biodegradation and or by coupling a suitable biofilteration technology in treatment system (Shan and Obbard, 2003). Controlling the concentration of un-ionized ammonia (NH_3) in the culture tank is a primary design consideration in recirculating systems. Ammonia must be removed from the culture tank at a rate equal to the rate it is produced to maintain a stable and acceptable concentration. In systems with external ammonia nitrogen treatment processes, the efficiency of the ammonia-nitrogen removal process will dictate the recirculating flow rate (Lorsordo *et al.* 1999). Ammonia removal from the culture system can be attained by using chemical filters, biological filters and by microbial amendments. In shrimp grow out systems under zero water exchange mode chemical and biological filters are practically and economically not viable. Hence, microbial amendments as bioaugmentors alone are considered here.

3.1.2 Microbial bioaugmentors.

Live bacterial inocula and other microbial amendments rich in extracellular enzymes are used in aquaculture (Boyd and Massaut, 1999) for various purposes including mitigation of total ammonia nitrogen (TAN), nitrite and nitrate (Gräslund and Bengtsson, 2001). Chuntapa *et al.* (2003) demonstrated the use of a cyanobacterium *Spirulina platensis* in shrimp culture for greatly reduced nitrogenous compounds at lower shrimp density. There was significant reduction in inorganic nitrogen compounds (NH_4^+, NO_2^- and NO_3^-), when a semi-continuous harvesting of *S. platensis* was adopted in shrimp culture ponds. But there are reports where commercial microbial products failed to register a significant difference in water quality between treated and control ponds (Shariff *et al.* 2001). TAN removal by biological nitrification can be achieved by maintaining optimal

conditions for the proliferation of sensitive chemoautotrophic nitrifying bacteria. Both bacterial groups involved in nitrification are obligate autotrophs, slow growers and have different sensitivities to environmental constraints such as salinity, light intensity and pH. Use of commercial nitrifying cultures is often limited as seed culture for the establishment of nitrifying bioreactors and biofilters. A commercial preparation called ABIL (ammonia binding inoculum liquid) was reported to be used as starter culture for shortening biofilter startup period and its efficacy was proved in fresh water aquaria systems (Grommen *et al.* 2002). Even though nitrifiers are present in the aquaculture system itself, bacterial amendments are useful as starter cultures which will rapidly acclimate and shorten the time for the development of fully operational biological filters. The time required to establish nitrification was shortened by about 30% of the time required without inoculation. Commercial nitrifying preparations are least studied on their ability to control TAN loadings under continuous TAN production. A commercial nitrifying culture (ABIL, Avecom, Germany) has been reported to increase rotifer population densities by a factor of 1.5 to 2.5 when added directly to batch cultures (Rombaut *et al.* 2003). Such nitrifying culture was used for the start up of bioreactors in rotifer batch cultures. The use of nitrifying culture (ABIL) as a starter culture in conventional $CaCO_3$ packed bioreactor improved water quality and rotifer growth (Rombaut *et al.* 2003). Fouratt *et al.* (2003) attempted to characterize a nitrifying bioaugmentation product (NBP, Novozyme, Salem, V.A. USA) using conventional and molecular techniques in a lab scale reactor, and found that increased nitrification activity was not correlating with shift in dominant organisms. However, literature is scanty in biostimulation and bioaugmentation of nitrification in grow out systems, especially that of shrimps.

3.1.3. Prospects of immobilized nitrifiers in aquaculture.

Both groups of nitrifiers are sensitive to conditions such as dissolved oxygen, pH, temperature, alkalinity and presence of toxic compounds (Sinha and Annachhatre, 2007). In the biological ammonia removal system of seawater, the nitrifying activity of bacteria has been reported to be extremely low due to the slow growth rate of nitrifying bacteria, the inhibition of nitrification by free ammonia and nitrite ions, and inherent high salt concentration. However immobilization techniques can be used to overcome these problems. (Seo *et al.* 2001).

Shan and Obbard (2001) demonstrated an in situ treatment method using clay pellets immobilized with nitrifying bacteria for the removal of TAN from marine shrimp

culture systems. It was reported that a lag phase of 4 days was observed for the onset of TAN removal when an immobilized marine nitrifying bacteria on clay pellets was applied in fresh water aquaria (Shan and Obbard, 2003). Recently biofilm formation of nitrifying bacteria on oyster shell surface, in a nitrification biofilter was studied by FISH and CLSM techniques (Ivanove *et al.* 2006). Tanaka *et al.* (2003) reported the use of photo cross linked beads in activated sludge unit for enhanced nitrification. It was found that bead associated organisms exhibited high specific activities under high loading conditions and their contribution to nitrification was significantly greater than suspended cells in the activated sludge unit. In another study, nitrifiers immobilized on polyacrylamide-gel carrier (Mitsubishi Reyon Co. Ltd., Japan) was used along with denitrifiers immobilized on macroporus cellulose (Aquacel, Taihoukougyou Co. Ltd., Japan) in a nitrification-denitrification single unit system (Nakano *et al.* 2004). Start up time for nitrifiers immobilized on macroporous cellulose and polyester carrier was one to two weeks. But 90-100% removal of ammonia-nitrogen was reported with short hydraulic retention time (Catalan-Sakairi *et al.* 1997). Nitrifying activated sludge from natural domestic sewage was entrapped in hydrogel beads in a study by Vogelsang *et al.* (2002) and found that gel bead ecosystem constituted a microbial community suitable for quantitative analysis and modelling due to its simple geometry and purely diffusive transport. Other biological nitrification techniques in sea water generally require long conditioning time to establish before operation. Biofilters which are commonly used in marine/ brackish water aquaculture invariably require one month for conditioning and can operate under a narrow range of TAN loadings. In the case of marine nitrifying sludge 'acclimated marine nitrifying sludge' (AMNS), two to three months of acclimatizing period was mandatory before operation (Furukawa *et al.* 1993).

Recently Gieseke *et al.* (2006) explored the possibility of less acidic microenvironments and/ or the existence of acid tolerant nitrifiers. Contrary to the notion that nitrifiers are sensitive to acidic conditions, attributed to the lack of substrate or toxicity of substrate with decreasing pH this study observed strong nitrification at pH 4.0 in biofilm grown on chalk particles. Biofilm grown on chalk particles was reported to support high rate nitrification at lower pH (Green *et al.* 2001). Inorganic carbon concentration, trace element concentration and the physiochemical properties of the carrier are important factors which affect nitrification in sea water (Catalan-Sakairi *et al.* 1997).

3.1.4. Advantages of immobilization

Bacteria in natural environments commonly exist attached to particulate material as biofilms, where their physiological properties may differ significantly from those of freely suspended cells. There is evidence for example, that biofilm populations have greater resistance to antibiotics and desiccation. Increased resistance to such factors may be due to the production of extracellular polymeric material, which is frequently associated with biofilm populations, or to differences in the specific growth rate of organisms within biofilms. Another handy feature is the re-usability of immobilized cells and its inherent ease of separating biomass from a system. Even dispersion of cells can be attained by the process of immobilization so as to tide over diffusional restrictions on the rate of reaction (Cheetham and Bucke, 1984).

Growth and activity of ammonia and nitrite oxidizing bacteria can be maintained in biofilms at pH values significantly lower than those required for growth of planktonic cells of nitrifying bacteria. Nitrifying biofilms are much more resistant to inhibitors of nitrification such as nitrapyrin and potassium ethyl xanthate due to the production of extracellular polymeric substances (EPS) (Batchelor *et al.* 1997). Efficacy of naturally occurring biofilms for ammonia removal has been explored by Thompson *et al.* (2002) in intensive shrimp culture systems. Biofilms consisting of pinnate diatoms, filamentous cyanobacteria and nitrifying bacteria reduced ammonia levels but there was higher output of nitrite and nitrate.

There is evidence that resuscitation of surface associated populations occurs more rapidly than that of freely suspended cells. This may be related to the phenomena of cell density signaling and bacterial communication. Close proximity and high concentrations of cells may therefore provide an ecological advantage to organisms in natural environments subjected to an intermittent substrate supply. Bacterial communication is via a cell-cell signaling system and accumulation of signal molecules, N- acyl homoserine lactones (AHL) (Batchelor *et al.* 1997). Cell densities of 10^{10}/mL occur only in biofilms and cell aggregates and if AHL is produced its diffusion from cells is reduced in biofilms.

The added beneficial feature of immobilized cell systems are the inert solid supports in dilute media may concentrate nutrients at the liquid solid interface and cells attached to these supports are exposed to higher nutrient concentration than existed in the bulk liquid. Thus higher cell populations faster growth rates and more rapid production rates may be achieved. Immobilized cultures tend to have higher activity and are more resilient to environmental perturbations than suspension cultures. Immobilization by encapsulation in

a matrix such as alginate and other specialized materials may be costly for water treatment, while surface immobilization on inexpensive materials like wood chip is a cheaper option (Shin *et al.* 2002). Natural materials used as carrier material for immobilization are inexpensive and compostable and once they are no longer useful as immobilization support they can be composted. Microbial mediated biodegradation is a problem while using materials derived from plant source containing more biodegradable fractions. Degradation of cellulose and hemicellulose leads to compromised mechanical integrity when microbes with elaborate hydrolytic enzyme system are immobilized. Moreover many synthetic materials like nylon fibres were inhibitory to growth (Shin *et al.* 2002).

More recently anaerobic ammonium oxidizing (anammox) bacteria were immobilized in polyethylene glycol gel carriers by entrapping a small amount of seed sludge in carrier (Isaka *et al.* 2007). Anammox bacteria immobilized in gel carrier increased the nitrogen removal activity to 3.7Kg N/m^3 reactor per day. In this case immobilization has overcome many practical constraints associated with the use of anammox bacteria for nitrogen removal like poor sludge retention, poor growth rate, and sludge floatation by intense bubble formation and long start up periods.

3.1.5. Immobilization

By way of immobilization it is expected that the microenvironment surrounding the immobilized cells is not necessarily the same experienced by their free cell counter parts. Shin *et al.* (2002) attempted immobilization of fungus *Trametes versicolor* ATCC 20869 on wheat straw, jute, hemp and maple wood chips for decolourization of textile dye amaranth. In general, colonization and growth of organism was more on natural media like jute and hemp rather than synthetic media based on polyethylene, polypropylene and nylon. In this experiment biomass did not attach well on maple wood chips. Porous ceramics carrier, sintered from a mixture of casting sand, zeolite and sawdust were used to immobilize microorganisms for waste water treatment packed bed bioreactors (Park *et al.* 1998). In denitrification bioreactors, to treat nitrate rich waste water, natural materials as structured biofilters media has been extensively studied. Wheat straw, saw dust, wood chip, shredded paper etc served as both packing material and carbon source for denitrification and their use and efficacy in bioreactors to treat nitrate rich waste streams arising from various point sources has been studied (Saliling *et al.* 2007). A wood based biofilter media to remove excess nitrate in septic tank effluent has been commercialized and marketed as Nitrex™. Saliling *et al.* (2007), reported the use of wood chips and wheat straw as

inexpensive alternative for packing media for denitrification processes for the treatment of aquaculture effluent and other high nitrate waste water. It was found that, wood chips and wheat straw gave comparable denitrification rates to commercial plastic media (Kaldnes^{TM}) but significant loss of mass, structural transformation and physical degradation were observed. Chitopearl a commercial carrier material (surface area 40-60m^2/g) with acetylized amino group of chitosan, having a chitin like framework; is available for immobilization (www.fujibo.co.jp).

Immobilization of nitrifying bacteria enhances nitrification, but they have limitations in the area of mass transfer of substrate and oxygen across the entrapment barrier. Oxygen consumption inside the gel matrix increases, due to increase in cell density and growth; thus rate of oxygen consumption easily overrides rate of oxygen diffusion across gel matrix. This may be manifested as oxygen deficiency in the core of gel matrix (Catalan-Sakairi *et al.* 1997) and results in poor nitrification rates. This effect has been exemplified by electron microscopy of microalgae *Chlamydomonas reinhardtii* immobilized gel beads, here the core of gel beads was poorly colonized due to diffusional limitation of nutrients (Vilchez *et al.* 1997). This was analyzed by scanning electron microscopy examination as it is a useful tool for studying biological viability of immobilized systems (Vilchez *et al.* 1997). This effect was similar to that reported in the case of *Nitrosomonas europea* immobilized on carrageenan (Wijffels and Tramper, 1989) and in the case of nitrifying activated sludge entrapped in hydrogels (Vogelsang *et al.* 2002). Philips *et al.* (2002) hypothesized the existence of oxygen limited autotrophic nitrification denitrification (OLAND) in nitrifiers, in order to tide over unfavorable conditions; ammonium is oxidized using nitrite as electron acceptor to form nitrogen gas under microaerophilic conditions. Ammonium can be removed as nitrogen gas by cooperating aerobic and anaerobic ammonium oxidizing bacteria (Vlaeminck *et al.* 2007a). Recently Vlaeminck *et al.* (2007b) demonstrated that reactivation of OLAND biomass is possible after long term storage at 4°C.

3.1.6. Immobilization methods.

Immobilization can be due to adsorption, covalent bonding, cross linking or it can be achieved by the techniques of entrapment and encapsulation.

3.1.6.1. Adsorption:

The adsorption phenomenon is based on electrostatic interactions (van der Waals forces) between the charged support and microbial cell. The actual zeta potential on both

of them plays a significant role in cell-support interactions. Unfortunately, the actual charge on support surfaces is still unknown and this limits the proper choice of supports for microbial attachment. Most surfaces have net negative charge. Along with charge on the cell-surface, composition of cell wall and nature of carrier will also play a predominant role.

3.1.6.2. Covalent bonding:

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (cross-linking) agent. For covalent linking, chemical modification of the surface is necessary. Apart from chemical cross linking, procedures employing physical processes such as flocculation and pelletization also benefit the immobilization techniques because of strong mutual adherence forces of some microbial cell cultures.

3.1.6.3. Entrapment:

The most extensively studied method in cell immobilization is the entrapment of microbial cells in polymer matrices. The matrices used are agar, alginate, carrageenan, collagen, gelatin, epoxy resin, photo cross linkable resins, poly acrylamide, polyester, polystyrene, polyurethane, cellulose and its derivatives.

3.1.7. Factors effecting immobilization.

Charge: Both ammonia oxidizing and nitrite - oxidizing bacterial cells exhibit negative charge during their growth period (Sousa *et al.* 1997). In Gram positive bacteria the thick wall is made up of cross-linked peptidoglycan covalently linked with highly negative charged polymers such as teichoic and teichuronic acids. The surface charge can be measured as electrophoretic mobility in a zeta-meter system.

van der Waals forces: Any suspended particle whether of an inorganic or biological nature are subject to van der Waals forces of attraction. Molecules in proximity experience attractive dispersion interactions and the dispersion energy (also called the van der Waals or electrodynamic energy).

Extra cellular polymeric substances: The extracellular polysaccharide or at least polymer formation by bacteria is involved in their ability to adhere to surfaces. It may simply form glue or it can be imagined that the polysaccharide molecules form hydrogen bonds with substances on the surface to which adhesion occurs. Alternatively, if it is an acidic polysaccharide, it may form ionic bonds with divalent metal ions involving a second array of negative charges on the surface.

Biomass concentration: The probability that a bacterium will encounter a submerged surface is dependent upon a number of factors. These include both the concentration of suspended cells and the time for which the suspension is exposed to the surface. An increase in either culture concentration or time has led to an increase in opportunity for attachment by increasing the probable number of bacterial collisions with the surface.

Motility: Another factor affecting the chance of encountering a surface is cell motility. Obviously, motile cells are more likely to meet a surface than are non- motile cells, which are dependent upon Brownian motion or water currents. Since most bacteria and most surfaces carry a net negative surface charge, repulsion forces must be considered important. Cell motility may also be important in that a motile bacterium may have sufficient kinetic energy to overcome electrostatic repulsion forces.

Hydrophobicity: Surface hydrophobicity plays a dominant role in bacterial adhesion because the van der Waals forces of attraction increases with the surface hydrophobicity (Sousa *et al.* 1997). The nitrifying performance of the biofilm formed onto polymeric supports (high density polystyrene, polyethylene, polypropylene, polyvinylchloride and polymethyl methacrylate) was correlated with the hydrophobicity and surface charge of both bacteria and support media. Polypropylene, the most hydrophobic material had the best properties for biofilm formation. The adhesion of nitrifying bacteria was mainly governed by hydrophobic interactions though electrostatic interactions when the supports had identical hydrophobicity. Surface properties of ammonia oxidizer and nitrite oxidizers are different; ammonia oxidizers have less hydrophobicity and smaller net negative charge whereas, nitrite oxidizers are more hydrophobic and less negatively charged. This fact can be responsible for the increase in the dissimilar behaviour of the supports concerning nitrite oxidation.

Apart from the above factors, properties of the substrate such as, hydrophilicity, steric hindrance, surface roughness and the existence of a conditioning layer at the surface are all thought to be important in the initial cell attachment process. Since most of the bacterial and support surfaces have a net negative charge, adhesion is controlled by some other interactions like hydrophobicity (Sousa *et al.* 1997). Immobilized cells can be divided into "naturally" attached cells (biofilm) and "artificially" immobilized cells. Naturally attached cells or biofilm is primary to waste water treatment processes. Biofilteration techniques make use of naturally attached cells. In aquaculture grow out

systems artificially immobilized cells of nitrifiers are essentially required for bioaugmentation.

3.1.8. Artificially immobilized cells

One of the most common techniques for artificial immobilization is gel entrapment. Materials that are widely applied for entrapment are alginate and carrageenan. For this, solution of polymers with cells suspended in them is extruded drop wise into a solution in which gelation of drops into solid spheres is initiated. In the case of alginate and carrageenan, gelation is initiated by Ca^{2+} and K^{+} respectively. The cells grow preferentially as a film just underneath the surface of the beads when cells have been artificially immobilized by gel entrapment in a gel. For this reason, substrate conversion rates tend to be proportional to the surface area and not to the total biomass.

With artificially immobilized cells it is also possible in principle to integrate nitrification and denitrification within a single biocatalyst particle. Because of the diffusion limitation of oxygen, the nitrifying organisms will concentrate in a film just under the surface of the gel particle leaving the anoxic central part completely unused. Denitrification may thus occur in the bead core if suitable denitrifying bacteria are present and if organic substrate is available.

Special equipment and time consuming works are required to prepare cells encapsulated in gel beads, and for this reason the immobilization of activated cells by entrapment is not economical and not practical. The use of suspended carriers to immobilize naturally occurring nitrifying bacteria by adsorption seems to be a more practical approach.

3.1.9. Characteristics of an ideal carrier

Void ratio:

Void volume is the volume that remains filled with air after the carrier media has been filled into a housing divided by the total carrier volume (Lekang and Kleppe, 2000). Low void ratio results in a longer contact time between the water and the established biofilm. The problems encountered with small void ratios include easier clogging, lower transport of air from the surface to the lower part of the filter and reduced water flow through the filter.

Specific surface area:

It is important that the carrier materials have a large surface area where biofilm can grow. A larger area allows more bacteria to grow per unit volume of filter medium, and results in increased removal of ammonia per unit of filter volume. Surface area can be calculated by BET (Brunauer, Emmett and Teller) gas adsorption isotherm method.

Density:

Carrier with density lower than that of water is preferable. If the carrier is heavier than water these particles will move downwards due to gravity. At the sediment-water interface organic carbon availability increases leading to anoxic conditions. Displacement of nitrifiers by heterotrophs will occur under these conditions. Since the nitrifiers are photo inhibitory care should be taken not to float the carrier-biofilm complex.

Economics:

Carrier must have a reasonable price. Most of the plastic media comes with a huge price tag which makes it less viable.

Homogeneous water flow:

To avoid dead zones and channels that will reduce the nitrification rate, it is important to have a carrier that gives the most homogeneous water flow possible.

3.1.10. Recent works on suspended-biofilm processes.

Aquacel (Macroporous cellulose carrier): Matsumura *et al.* 1997. This carrier "AQUACEL" is made of foamed cellulose with continuous macropores. This structure is intended to allow forced convection of the liquid inside the carrier resulting in increased oxygen transfer rates. This increase in oxygen transfer rate leads to high cell concentration inside the carrier. The surface of the aquacel was treated with polyethyleneimine (PEI) to provide a positive charge and an ion exchange capacity (IEC) of 0.8- 1.3 meq/g and stabilized by cross linking. The effective surface area is high (3-7m^2/g), pore size 500μm, a void fraction of 97% and an apparent density of about 1.05 Kg.wet./dm^3. To immobilize nitrifying bacteria a desired amount of dried aquacel was soaked in seawater for six hours. During this time, the carriers swelled, and the bacteria were trapped in the carriers. The seawater was replaced with fresh synthesized medium, and then bacterial activation was

carried out. Nitrification rate was found to be 100% at a loading rate of 12 Kg-N/m^3 carrier/day.

Crushed leca (Dried expanded clay): Lekang and Kleppe, 2000. Leca is the commercial name of light weight clay aggregate (LWA) which is a clay product that is dried, expanded and formed into small balls under industrial conditions. There have been few measurements made on the surface area of leca. Exterior specific surface areas between 500 and 1000m^2/m^3 have been measured. Nitrification rate (100%) was between 0.1 and 0.2g TAN/m^2/day.

Biopop: Kim *et al.* (1997) reported the use of a novel modified cellulose carrier for nitrification, which was developed through thermodynamic analysis. In this work various carrier materials were analyzed for surface free energy and surface tension by contact angle measurement and finally a relationship between free energy change and nitrification rate was established (Kim *et al.* 1997). By measuring the contact angles among nitrifying bacteria, solid carrier material and broth, a change in free energy of adhesion of nitrifying bacteria on carrier was calculated. In this study cellulose gave the most favourable result and all nitrifying bacteria attached to the carrier material within one day and it gave a nitrification rate of 800g N-NH_3/m^3day (Kim *et al.* 1997). Biopop made of cellulose showed a stronger polar nature than other carriers tested like 'linpor' (made of polyurethane), 'biosac' (rubber coated with activated carbon) and natural rubber. They also reported that nitrifying bacteria cultured using inorganic salt medium showed strong polar nature compared with other microbes cultured with surfactants.

3.1.11. Immobilization of nitrifying bacterial consortia on an appropriate support material and development of bioaugmentor

Having all described above at the background, a research programme was undertaken to immobilize four nitrifying bacterial consortia (NBC) such as AMOPCU – 1, NIOPCU – 1, AMONPCU – 1 and NIONPCU – 1 on an appropriate support material and to develop as bioaugmentors for applying in zero exchange shrimp grow out systems. The following pages summarises how this task was accomplished.

3.2. Materials and methods.

3.2.1. Immobilization of nitrifying consortia.

Four nitrifying bacterial consortia (NBC) such as AMOPCU-1, NIOPCU-1, AMONPCU-1 and NIONPCU-1 were chosen for developing appropriate bioremediation programmes in terms of bioaugmentation in shrimp grow out systems. As it had been demonstrated that nitrifying bacteria perform better under the state of immobilization (Seo *et al.*, 2001), the option was to standardize a technique which is user friendly and appropriate for grow out systems.

3.2.1.1. Selection of suitable carrier material

For applying in aquaculture systems the carrier material for the NBC should have several unique features:

- Environment friendliness: The carrier must be biodegradable over a period of time with out leaving any residue.
- High void ratio.
- Large specific surface area where biofilm can grow: A larger area allows more bacteria to grow per unit volume/area of carrier and results in increased removal of ammonia.
- Density: Must be almost similar to that of water
- Homogeneous water flow: Surface profile of the carrier should be such that it allows homogeneous water flow.
- Economics: Should have a reasonable price.

3.2.1.2. Selection of wood powder as an ideal carrier material.

To satisfy the above requirements it was decided to select natural materials preferably polymers for immobilization. Accordingly wood powder from four woody plant species such as *Ailanthus altissima, Macaranga peltata, Hevea braziliensis, Mangifera indica* and chitin extracted from prawn/shrimp shell waste were screened as the support material for immobilizing the NBC.

3.2.1.3 Processing the carrier materials.

Wood chips of the respective plant species were collected from local timber industries and chitin flakes from M/s India seafoods, Private limited, Kannamaly, Kochi.

They were dried, crushed and sieved to get particle size ranges such as 300-500, 500-710, 710-2000, 2000-2500, 2500-3000 and 300-1500μm used for the experiment. As lignin in wood powder might interfere with the process, it was delignified and its suitability as the carrier material investigated with all the four NBC.

For delignification the method proposed by Wood and Saddler (1988) was employed as follows: 1g of crushed wood powder was immersed in 50mL distilled water containing 1% v/v H_2O_2. With 0.1 N NaOH, pH of the suspension was adjusted to 11.5. The suspension was stirred gently at 25°C for 18-24 hrs on magnetic stirrer, with hourly correction of pH to 11.5 as per requirement. The suspension was filtered and the insoluble residue collected, washed with distilled water, till pH dropped to neutrality. Delignified wood residue was dried at 100°C before storage. Presence of lignin residue was checked by chlorinating the delignified tissues followed by treatment with hot sodium sulphite. To 0.5g delignified material 5mL 4% (w/v) sodium hypochlorite and 5mL aqueous hot sodium sulphite (5g/50mL) were added. Release of magenta coloured liquor indicated the presence of lignin.

However, the method required optimization to arrive at the lowest percentage of H_2O_2 required and duration of exposure for the total release of lignin. Accordingly the experiment was conducted with 0.2, 0.4, 0.6, 0.8 and 1% H_2O_2 (v/v) with varying time intervals. In another set of experiment distilled water was replaced with tap water. Efficacy of the methods was evaluated following the protocol described above.

3.2.1.4. Screening for the most appropriate substratum for immobilization

Selection of the most appropriate material from the collection of wood powder drawn from the four woody plant species, and chitin a series of screening experiments were carried out.

a) Determination of surface profile: Surface profile of the support materials was analyzed by scanning electron microscopy. Wood powder samples and chitin having particle size 300 - 500 μm and 500 - 710 μm were spread on electron microscope stubs, dried in critical point drying apparatus, gold coated and observed under a scanning electron microscope (Leo 435 VP SEM, UK).

b) Surface area measurement: two dimensional areas of wood powder samples having different size ranges were calculated by image analysis of the scanning electro-micrographs (Soft Imaging Viewer, Soft Imaging System GmbH, version 3.1, build 507).

Based on the above two analyses, specific surface area measurement of the substratum drawn from *A. altissima* was accomplished following Brunauer-Emmett-Teller (BET) analysis. Small diameter media usually have a higher specific surface area and a lower void ratio than those larger diameter media of the same type. Specific surface area is usually a function of the media type selected. The higher the specific surface area, more bacteria can grow in a unit volume, and greater will be the total ammonia removal efficacy. Surface area was measured by Brunauer-Emmett-Teller analyzer (BET analyzer: Quantachrome NOVA automated gas sorption system, Quantachrome instruments).

3.2.1.5. Activation of the nitrifying bacterial consortia.

As the NBC are stored at 4°C it was necessary to bring them back to room temperature and activate before immobilization. An aliquot of 10mL each from each of the consortia were transferred to 1000 mL Watson's medium (1965) supplemented with NH_4^+-N in the form of NH_4Cl in NO_2^--N in the form of KNO_2 in 2L conical flasks in two salinities, 15 and 30. The flasks were well aerated from an air pump at a rate of 2L/min through a cartridge filter (Sartorius India Pvt. Ltd.) and incubated under obscurity. Daily monitoring was done to check the substrate consumption and product formation. Whenever there was lowering of pH, it was adjusted to the optimum (7.0 to 8.0) using sodium carbonate. Addition of sterile distilled water compensated evaporation loss. As the substrate consumption progressed, it was supplemented with fresh aliquots of NH_4^+-N/ NO_2^--N to maintain the concentration at 10µg/ml.

3.2.1.6. Cell dispersion of nitrifying bacterial consortia and determination of cell count.

Quantitative determination of NBC 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). Accordingly, nitrifying bacterial consortia equivalent to 0.01g wet weight were subjected to different ultrasonic treatments for cell dispersion employing an ultrasonicator (Vibra cell, Sonics, USA) at different power outputs such as 100, 125 and 150W and duration as 1,2,3,4 and 5 min. Cell dispersion was evaluated by counting the number of single cells generated through each of the treatments by epifluorescence microscopy.

Sonicated bacterial samples were stained with acridine orange and filtered through 0.2µm Irgalan black stained nuclepore 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, nuclepore filters were mounted immediately on a slide using non-fluorescent immersion oil. A minimum of seven fields per filter per sample were counted. Bacterial cells fluoresced green taking up acridine orange under blue excitation on an epifluorescence microscope (Olympus CX-41). Fluorescing images less than 0.2μm in diameter were disregarded. An eyepiece of known area was used during enumeration.

Calculation

Bacterial abundance (cells/liter) = $C_f R$ x 1/Fs

C_f = mean number of cells /field.

R = active area of filter/ area of field counted.

Fs = volume of fluid filtered (litres).

3.2.1.7 Immobilization of the nitrifying bacterial consortia on the support materials.

Carrier materials selected for immobilization were - Delignified wood powder drawn from *Ailanthus altissima* (particle size 300-500μm and 500-710μm); *Macaranga peltata* (particle size 300-500μm and 500-710μm); *Hevea braziliensis* (particle size 300-500μm and 500-710μm); *Mangifera indica* (particle size 300-500μm and 500-710μm) and Chitin powder (particle size 300-500μm and 500-710μm). Each type of the carrier material was used for immobilizing the NBC listed below.

Nitrifying bacterial consortia used in the study.

S.No	Nitrifying bacterial consortia	Name
1	Ammonia oxidizing consortia for non-penaeid culture systems.	AMONPCU-1
2	Nitrite oxidizing consortia for non-penaeid culture systems.	NIONPCU-1
3	Ammonia oxidizing consortia for penaeid culture systems.	AMOPCU-1
4	Nitrite oxidizing consortia for penaeid culture systems.	NIOPCU-1

The carrier materials were weighed to get equal surface area (calculated based on the 2D image analysis) and transferred to tubes containing 25ml Watson's medium (1965). The tubes were inoculated with AMONPCU-1 and NIONPCU-1 having $1x10^7$ cell density and AMOPCU-1 and NIOPCU-1 having $1x10^6$ cell density, aerated from an air pump at a rate of 0.25L/minute and incubated under obscurity. Nitrification was checked in terms of

TAN consumption and NO_2^--N production in the case of ammonia oxidizers and NO_2^--N consumption and NO_3^--N production in the case of nitrite oxidizers. Drop in pH was adjusted using 1% sodium carbonate and evaporation loss made up with sterile distilled water. Whenever NH_4^+-N/ NO_2^- -N got depleted, substrate addition was resorted to get a final concentration of 10mg/L. After incubating for 10 days the carrier materials were gently washed in the medium and transferred to fresh one and the same analysis was continued, to record the nitrifying potency of immobilized nitrifying consortia.

To confirm and document the extend of adhesion of nitrifying consortia to wood powder, scanning electron microscopy was performed following the procedure described below. The substrata with immobilized nitrifiers were washed with sterile seawater of respective salinity and fixed in 2.5% gluteraldehyde prepared in seawater at 4°C overnight. The pellets were washed and post fixed in 2% osmium tetroxide at 4°C for 2 hours. Subsequently, these particles were washed repeatedly with seawater and dehydrated. The dehydration was done through an acetone series of 70 -100%. The particles were spread on SEM stubs, dried in critical point drying apparatus, gold coated and observed under scanning electron microscope (Leo 435 VP SEM, UK).

3.2.1.8. Preferential selection of particle size range of the substratum (wood powder from *Ailanthus altissima)* for enhanced nitrifying potential.

Based on the experiments conducted as described above the woody plant *Ailanthus altissima* (Pongalyam) was segregated as the most appropriate species from among the tested varieties as the source of the substratum. Wood powder from the plant having particle size 500-710µm, 710-2000µm, 2000-2500µm, 2500-3000µm and 300-1500µm was subjected for an experiment to select the ideal particle size to be used further. The experiment was conducted by using two sets of materials, one set with equal surface area (calculated from the 2D area obtained from image analysis) and the other with equal weight. Materials (0.1g each) were weighed, sterilized and transferred to 25ml medium and inoculated with the NBC (1mL each of AMONPCU-1 and NIONPCU-1 having $1x10^7$ cell/mL and AMOPCU-1 and NIOPCU-1 having 1 x 10^6 cell/mL). Tubes were aerated, incubated under obscurity and monitored daily for nitrification and TAN and TNN removal per day.

3.2.1.9. Nitrifying potency of the consortia immobilized on wood powder

NBC immobilized on wood powder was transferred to fresh medium and nitrification was examined as described above.

3.2.1.10. Time course of immobilization of nitrifying consortia on the substratum.

Suspensions (100mL aliquots) of NBC ($1x10^{10}$cells/mL) were incubated with 1g autoclaved substratum (particle size 300-1500μm) at 28-30^{0}C for all the consortia in individual conical flasks with an initial NH_4^+-N & NO_2^--N concentrations of 10mg/L each. The flasks were incubated in obscurity under aeration at a rate of 1L/minute through cartridge filters, pH maintained between 7.0 and 8.0. The preparation was kept under suspension for 6, 12, 24, 48 and 72h with periodic analysis of pH, TAN, NO_2^--N, and NO_3^--N. On 90% depletion of TAN/TNN in the medium additions were made to get a final concentration of 10mg/L. Wood powder samples were transferred after 6, 12, 24, 48 and 72h to different micro centrifuge tubes and fixed for scanning electron microscopy analysis as per the protocol mentioned above.

3.2.1.11. Determination of the minimum number of cells required for immobilizing unit weight (1g) substratum.

Adopting the method of immobilization as described above, the experiment was conducted to determine minimum cells of NBC required for immobilizing 1g substratum (Particle size 300 -1500μm) having a surface area of 1.87m^2/g. To 100 mL Watson medium (1965) flasks containing 1g substratum (dry weight), nitrifying bacterial consortia were inoculated so as to get 10^5, 10^6, 10^7 and 10^8cells/mL. The cell count was determined by epifluorescence microscopy after sonication for cell dispersion. Nitrifying activity during and after immobilization was measured in terms of substrate (TAN/TNN) consumption and product formation (NO_2^- and NO_3).

3.2.1.12. Determination of the quantity of immobilized nitrifying bacterial consortia required for treating unit volume of seawater.

Activated NBC (AMONPCU-1 and AMOPCU-1) having 10^{10}cells/mL) in 500mL aliquots were aerated with sterile wood powder (10g) for immobilization and maintained for three days with periodic correction of pH and addition of the substrates. After three days, 0.2, 0.4, 0.6, 0.8, and 1.0g each of the preparations were administered to FRP tanks, in duplicates, having 20L seawater (salinity 15 and 30 respectively) with aeration. Control

tanks were with sea water having the substratum alone. Nitrification over a period of 3 days was monitored and measured in terms of TAN consumption and NO_2-N/ NO_3-N production.

3.2.1.13. Nitrification potential of immobilized nitrifiers on storage

To determine the nitrification potential of the immobilized nitrifiers after one month storage, aliquots of the preparations (0.1g) were examined initially for TAN and TNN removal by incubating in 50mL Watson's medium (1965) under aerated conditions. Subsequent to storage at room temperature in minimal quantity of the medium in tightly closed tubes. From this preparation 0.1g aliquots were removed and the TAN and TNN removal rates were measured by determining the initial and final TAN and TNN values for a period of 72hrs.

3.2.1.14. Denitrification potency of the consortia.

During experiments with NBC, either with or without immobilization, total removal of TAN/ and NO_2-N was observed with lesser/ or no product (NO_2-N/ NO_3-N) formation. It is hypothesized that such a situation normally happens, if the consortium resorts to denitrification or/ and their assimilation as nitrogen source. To test the hypothesis an experiment was designed to evaluate the denitrifying potential of the consortium employing the test of nitrate reduction. This was carried out by inoculating the NBC in Watson's medium (1965) supplemented with 0.1% KNO_3. They were incubated until good growth was obtained during which time samples were examinced periodically to detect the products of denitrification such as TAN, NO_2^--N and N_2 besides NO_3^--N.

3.2.1.15. Evaluation of the nitrifying potency of immobilized NBC in a bioassay system.

The experiment was conducted in three different salinity regimes, such as 10, 20 and 32. The consortium AMONPCU-1 was evaluated at salinity 10 and 20 and AMOPCU-1 at 32. The experimental design consisted of 12 tanks, each holding 24L seawater, maintained under aeration at a rate of 2L/minute. Each set consisted of a control and 3 replicates. The experiment was conducted having 3 shrimps in each tank with an average weight of 15g (*P. monodon*) maintained without water exchange and fed with commercial shrimp pelleted feed at a rate of 4% of body weight. After 8 days when TAN loadings were high, 3g (wet weight) immobilized nitrifying consortia were applied per tank. Water quality parameters such as, TAN, NO_2^--N, NO_3^-N, alkalinity, pH, BOD, turbidity and

temperature were monitored daily. Extend of degradation of wood powder used as substratum was also evaluated daily by physical examination.

3.2.1.16. Design and fabrication of a simple device for mass immobilization of AMOPCU -1 on the substratum.

For immobilization of NBC a simple device (Fig 3.1) was designed and fabricated in FRP. The cylindrical device with conical tapering bottom has a capacity of 50L and is fitted with a stirrer assembly and an air diffuser at the bottom.

To 20L seawater (salinity 30), 10mg/L NH_4^+-N and 2mg/L PO_4-P (as NH_4Cl and KH_2PO_4) were added, pH adjusted to 7.5 using Na_2CO_3 and inoculated with AMOPCU -1 to get 10^7cells/mL. 400g delignified wood powder from *Ailanthus altissima* as the substratum was added to the immobilization tank and aeration set at 6L/minute. Samples were analyzed daily for pH, TAN, NO_2^--N and NO_3^--N. On depletion of NH_4^+-N by 90% additions were made to bring up the concentration to 10mg/L. Activity in terms of TAN consumption and NO_2-N/ NO_3-N production was monitored. During the process 3 samples were drawn daily, washed gently in sterile medium (Watson's 1965) and transferred to fresh one for analyzing nitrifying potency as described above.

3.2.1.17. Evaluation of the potency of immobilized nitrifying bacterial consortium in shrimp grow-outs.

The NBC immobilized on the substratum was administered in shrimp grow out system when there was high TAN detected during routine analysis. The immobilized nitrifier was applied uniformly in the culture ponds at the rate of 400g/ hectare after diluting with pond water. Water quality parameters such as TAN, NO_2^--N, NO_3^--N, alkalinity and pH were measured subsequently.

3. 2.1.18. Immobilized NBC on the substratum drawn from *A. altissima*

Based on the protocol developed the NBC on immobilized state has been generated and examined under scanning electron microscope following the procedure described above. This culminated in the development of a product for bioremediation of ammonia toxicity in shrimp grow out system. The product has been named TANOX (Total Ammonia Nitrogen Oxidizer).

3.3. Results.

3.3.1. Processing the carrier materials

The carrier materials, especially wood powder from four plant species, identified for immobilizing NBC required processing to get rid of lignin which might interfere with the immobilization and subsequent nitrification. This was accomplished by treating with H_2O_2 in alkaline pH. Efficacy of the delignification protocols implemented is summarized in Table 3.1. The results indicated that the treatment with 1% (v/v) H_2O_2 in tap water for a period of 3 to 5 hrs at pH 11.5 oxidized lignin to the point of no release subsequent to chlorination (Fig. 3.2).

3.3.2. Determination of surface profile

Surface profile of the substrata was documented and evaluated by SEM. The electron micrographs generated from 4 woody plant species and chitin in two ranges of particle sizes are presented in Fig. 3.3. Among the samples examined delignified *A. altissima* (300-500μm and 500-710μm) particles exhibited the largest number of corrugations suggesting comparatively more surface area available for attachment.

3.3.3. Specific surface area.

Specific surface area of the substrata was measured in two stages. Primarily two dimensional surface areas of the samples having different size ranges were calculated by image analysis of the scanning electron micrographs (Fig. 3.3) as presented in Table 3.2. Among the four species of woody plants examined the particles of *A. altissima* having size ranges, 300-500μm and 500-710μm, were found to have the highest surface area (0.7 and 0.6 m^2/g respectively).

In the next stage of surface area assessment, the particles of *A. altissima* were subjected for BET analysis to get the absolute specific surface area required for all further experiments. Based on this analysis they were found to have 1.87m^2/g.

3.3.4. Activation of nitrifying bacterial consortia.

Prior to immobilization the NBC were activated. This was carried out by transferring an aliquot of 10 mL each of the consortia stored at 4^0C to 1L Watson's medium (1965) supplemented with 10 ppm NH_4^+-N/ NO_2^--N and incubating under aeration (2L/minute) continuously for a week in obscurity. At the end of the incubation

period, average NH_4^+-N consumption was 4.0 ± 0.53 mg/L/day by AMONPCU-1 and 6.2 ± 0.78 mg/L/day AMOPCU-1. In the case of nitrite oxidizing consortia, substrate consumption was 5.37 ± 1.2 mg/L/day for NIONPCU-1 and 6.89 ± 0.98 mg/L/day for NIOPCU-1 respectively. In all the 4 consortia products of nitrification were either absent or below detectable limit.

3.3.5. Cell dispersion of nitrifying bacterial consortia and determination of cell count.

In order to facilitate enumeration of cells in the consortium, dispersion of the former was required. This was achieved by ultrasonication which was performed at different power outputs and duration. On enumerating the ultrasonicated samples by epifluorescence microscopy, the highest number of cells obtained was in the treatment regime at 125W for 4 min as presented in the Fig 3.4. Samples treated at 100 W for 1 to 5 minutes and at 125 W for 1 to 3 min yielded poor results, as clumps / aggregates were visible in the preparations and hence, those samples were not subjected for enumeration.

3.3.6. Immobilization of the nitrifying bacterial consortia on support materials.

Even though all carrier materials were found to support nitrifying bacterial consortia, it was essential to select one for immobilizing all the four consortia for practical purposes. This selection was based on the consistency in nitrification observed by incubating the NBC with all types of substrata and the same with the immobilized consortia on the substrata; the consistency expressed in terms of coefficient of variance (Cv). Among all types of substrata experimented the one derived from *A. altissima* with particle size 300 -500 & 500-710µm was found to have the least coefficient of variance (Cv) (7.03 and 5.8%) (Table 3.3 and 3.4).

3.3.7. Effect of particle size of the substratum (*Ailanthus altissima*) in nitrification.

As the next step, the most ideal particle size of the support material was selected by way of preferential attachment of the NBC. The substrata in five particle size ranges derived from *A. altissima* were used for immobilizing all the four nitrifying bacterial consortia and the rates of nitrification in fresh medium were assessed (Fig 3.5 and 3.6). TAN and TNN removal by the consortia immobilized on the substratum in different particle size was not significantly different among the particle size ranges tested ($p>0.05$). When transferred to fresh medium after immobilization, substratum derived from *A.*

altissima with particle size 300-1500μm showed better performance in removing TAN and TNN.

3.3.8. Time course of immobilization of nitrifying bacterial consortia.

Scanning electron microscopy revealed the progression in the development of biofilm of nitrifying bacterial consortia on the substrata (Fig 3.7, 3.8, 3.9 and 3.10). In general in all situations attachment of cells could be seen at 12^{th} hour exposure of the substratum to the consortia. On the 24^{th} hour of incubation progression in the biofilm formation could be seen however, on the 48^{th} hour disintegration of the substratum was evident which progressed to 72^{nd} hour.

3.3.9. Determination of the least number of cells required for immobilizing on unit weight/area of substratum.

NBC were used at a cell count of 10^5, 10^6, 10^7 and 10^8 per mL for immobilizing on 1g substratum (area 1.87m^2/g) suspended in 100 mL Watson's medium (1965). After three days, the substrata were removed to fresh medium and nitrification determined. Average TAN removal rate of AMONPCU-1 having 10^7 cells/mL as initial cell count was found to be the highest (1.33 ± 0.25mg/day/m^2) and on resuspending the immobilized material in fresh medium it gave an activity of 0.41 ± 0.09mg/day/m^2 (Fig 3.11). Cell count higher than this (10^8 /mL) had no additional effect on increasing TAN removal. The results indicated that 10^7 cells/mL would be sufficient as the initial cell count required for initiating effective immobilization on 1g substratum suspended in 100mL medium. Overall TAN removal rates were significantly different among the treatments having cell counts ranging from 10^5, 10^6, 10^7 and 10^8 per mL during immobilization (p 0.002226) but insignificant when its activity was tested in fresh medium (p 0.664933) as per single factor ANOVA.

In the case of AMOPCU-1 maximum TAN removal of 1.73 ± 0.133mg/day/m^2 was observed in the treatment with an initial count of 10^7 cells/mL. When this product was tested in fresh medium TAN removal obtained was 0.395 ± 0.08mg/day/m^2. Statistical analysis with single factor ANOVA showed that there was no significant difference between the treatments with the cell count of 10^5, 10^6, 10^7 and 10^8 per mL during immobilization in 1g substratum (area 1. 87m2/g) (p 0.294989). Same was the situation when the activity was tested in fresh medium (p 0.073377). Above results suggest that 10^5

to 10^8 cells per mL in 100 mL immobilized in 1. 87m^2/g does not make any difference in the final activity.

3.3.10. Determination of minimum quantity of immobilized nitrifying bacterial consortia required for treating unit volume of seawater.

Immobilized nitrifying consortia were prepared by incubating 10g substratum having particle size 300 to 1500μm with 5 x 10^{12}cells of consortia in 500 mL Watson's medium (1965) under aeration (2L/minutes) for three consecutive days. Nitrification was monitored in terms of per day TAN consumption and NO_2^--N / NO_3^--N production. (Table 3.5). This material was used for determining the minimum quantity of immobilized NBC required for establishing nitrification in unit volume water.

To accomplish the above, varying quantities of the above material was incubated in 20 L seawater having salinity 15 and 30 respectively in FRP tanks and nitrification monitored in terms of per day TAN consumption and NO_2-N/ NO_3-N production. On day 3, per day TAN removal was 0.61, 0.76, 0.64, 0.96, and 1.2 ppm, in seawater having salinity 15 inoculated with 0.2, 0.4, 0.6, 0.8, and 1.0g NBC (AMONPCU-1) immobilized substratum respectively. Whereas in the control flasks it was 0.19 ppm. In the flasks with sea water having salinity 30 treated with 0.2, 0.4, 0.6, 0.8, and 1.0g NBC (AMOPCU-1) immobilized substratum, per day average TAN removal was 0.66, 0.95, 1.21, 1.2 and 1.03 ppm respectively having only 0.38 ppm removal recorded in the control tanks. Results are summarized in Table 3.6. TAN removal per day and the amount of wood powder with immobilized consortia exhibited positive correlation on the third day (correlation coefficient 0.931521 and 0.831977 respectively). But nitrite production in the treatment with AMONPCU-1 a high positive correlation on day 1 (r 0.847697) was registered with subsequent progressive lowering on day 3 (r 0.365003). AMOPCU-1 showed better correlation (r 0.82215) between quantity of wood powder and nitrite production on day 3. Precisely it indicates that higher the quantity of the immobilized NBC greater will be the nitrification in a treatment system. Nevertheless, ANOVA with in a set of different quantity of the immobilized material showed no significant variation in nitrification.

3.3.11. Nitrifying potency of immobilized consortia and its shelf life.

Soon after immobilization nitrifying potency of the preparation was tested in fresh medium in conditions identical to those used for immobilization. The average TAN removal rates of immobilized wood powder were 0.53 ± 0.16 mg TAN/m^2/day in the case

of AMONPCU-1 and 0.57 ± 0.14 mg TAN/m^2/day for AMOPCU-1. Nitrite removal rates for immobilized NIONPCU-1 and NIOPCU-1 were 0.81 ± 0.27 mg NO_2-N/ m^2/day and 0.86 ± 0.2 mg NO_2-N/ m^2/day respectively. The maximum TAN removal rate of freshly immobilized wood powder was 0.69mg TAN/m^2/day and nitrite removal at a rate of 1.08mgNO_2-N/ m^2/day.

Immobilized nitrifying consortia were stored at room temperature in sterile plastic bottles, immersed in liquid media. Bottles were sealed prior to storage in dark for one month. After one month TAN removal rate and NO_2-N removal rate were measured with and without aeration. Results (Table 3.7) showed that storage did not hamper nitrification potency, but TAN removal rates were lower than the one recorded for freshly immobilized materials (Fig 3.12).

3.3.12. Denitrification potency of the consortia.

In almost all experiments TAN removal was nearly complete. Meanwhile, to assess the potency of denitrification, NO_3 - N removal was monitored at different time intervals in media containing 10ppm nitrate under both aerobic and anaerobic conditions (Table 3.8). Under anaerobic condition NO_3 - N removal was well correlated with NO_2^- - N production with a correlation coefficient of 0.9997. However, under aerobic conditions positive correlation was not obtained in nitrate removal and nitrite buildup; but TAN production was well correlated with NO_3 - N utilization with a correlation coefficient of 0.9988.

3.3.13. Evaluation of nitrification potency of immobilized nitrifying consortia in a simulated bioassay system.

TAN removal was obvious from third day onwards (Fig 3.13). After 2 days, average TAN removal in the tanks having sea water with salinity 32 was 3.61 ppm, twice the rate observed in the corresponding control. Average lowering of 2.97 ppm in TAN was observed in the tanks with sea water having 20 salinity after 24 hours. Whereas in the corresponding control a lowering of 1.46ppm, half the removal observed in the test tanks, was observed. In the tanks with seawater having salinity 10, average TAN removal was 4.18 ppm and the control exhibited a removal of 2.87ppm.

TAN removal and drop in alkalinity in tests (Fig. 3.14) were positively correlated (r = 0.9171, 0.9584 and 0.9551) in the systems with seawater having salinity 10, 20 and 32ppt respectively. Moreover, decrease in alkalinity and resultant drop in pH (Fig. 3.15)

exhibited strong correlation in the experimental tanks (r = 0.9741, 0.8797 and 0.8802) in the systems with seawater having 10, 20 and 32 salinity) than in the control tanks (r = 0.9181, 0.7737 and 0.6888). In all tests NO_2 - N registered two fold increase after day 2nd (Fig. 3.16), which declined corresponding to the decline of TAN concentration. Nevertheless, NO_3^- - N was not detected or was below detectable level through out the experiment. Application of the substratum with immobilized NBC did not reflect in the BOD, which was well below 50 mg/L (Fig. 3.17) and there was no increase in turbidity also. Meanwhile in the control tanks the substrata with out consortia were readily degraded increasing the bottom detritus. Physical appearance of the substratum was different in control and experimental tanks; it was blackened in control tanks with little degradation and having no colour change (Fig. 3.18) in the experimental tanks.

3.3.14. Mass immobilization of AMOPCU - 1 on the substratum.

Nitrifying consortium was immobilized on the particles of *A. altissima* in a mass immobilizing device (Fig. 3.1). When AMOPCU - 1 was immobilized on the substratum with particle size 300-1500µm, average TAN removal observed was 3.5 ± 1.52 mg/L/day. NO_2 - N and NO_3 - N concentrations were below detectable limits. Immobilized wood powder sampled on day 4 and 5, exhibited substantial TAN removal at the rate 5.67 ± 1.6 mg/L/day (0.3 ± 0.085 mg/day/m^2) and 5.23 ± 1.91 mg/L/day (0.28 ± 0.1 mg/day/m^2) respectively, in fresh medium. To compare whether there is any significant difference between the TAN removal rate by the samples collected on day 4 and 5, single factor ANOVA was executed. Statistical analysis proved that there was no significant increase in TAN removal rate after 4 days (*p value* > 0.05) (Table 3.9).

3.3.15. Evaluation of mass immobilized nitrifying consortia in shrimp grow-out ponds.

Immobilized NBC (AMOPCU - 1) was administered in shrimp pond where sudden high TAN loadings were reported. TAN loading was brought down by half (from 1ppm to 0.5ppm) with in 24hr in a one hectare shrimp culture pond. Water quality of test and control ponds is summarized in Table (3.10).

3. 3.16. Immobilized NBC on the substratum drawn from *A. altissima*

The scanning electron micrographs presented in Fig. 3.19 demonstrate the attachment of the NBC on the selected carrier material. This is the final form of the

product generated ready for application meant for delivering nitrifiers into shrimp grow out system.

3.4. Discussion

Shrimp grow out systems are specialized and highly dynamic aquaculture production units which when operated under zero exchange mode require bioremediation of ammonia and nitrite nitrogen to protect the crop. The research conducted here is to develop an economically viable and user friendly technology for addressing the problem of ammonia toxicity in the aforementioned system. The NBC generated earlier (Achuthan *et al.*, 2006) were used for developing the technology. As these consortia form biofilm, it was decided to develop a simple, less expensive, environment friendly delivery system for application. As biodegradable carriers are required for the above which will not leave any residue on degradation, wood powder from four woody plants common in the region, and chitin from prawn shell waste were selected for screening and selecting the best.

Bacteria in natural environments commonly exist attached to particulate material as biofilm, where their physiological properties may differ significantly from those of freely suspended cells. There is evidence for example, that biofilm populations have greater resistance to antibiotics and desiccation. Increased resistance to such factors may be due to the production of extracellular polymeric materials, which is frequently associated with biofilm populations, or to differences in the specific growth rate of organisms within biofilm. Another handy feature is the re-usability of immobilized cells and its inherent ease of separating biomass from a system. Even dispersion of cells can be attained by the process of immobilization so as to tide over diffusional restrictions on the rate of reaction (Cheetham and Bucke, 1984).

Growth and activity of ammonia and nitrite oxidizing bacteria can be maintained in biofilm at pH values significantly lower than those required for growth of planktonic cells of nitrifying bacteria. Nitrifying biofilms are much more resistant to inhibitors of nitrification such as nitrapyrin and potassium ethyl xanthate due to the production of extracellular polymeric substances (EPS) (Batchelor *et al.* 1997). Efficacy of naturally occurring biofilms for ammonia removal has been explored by Thompson *et al.* (2002) in intensive shrimp culture systems. Biofilms consisting of pinnate diatoms, filamentous cyanobacteria and nitrifying bacteria reduced ammonia levels but there was higher output of nitrite and nitrate. However, the main concern in this work has been to identify an appropriate carrier material for application in shrimp grows out systems.

Since wood powder from four woody plants have been identified as the possible carrier material along with chitin, at the outset, a protocol for delignification was standardized as lignin was thought to interfere with immobilization. According to the protocol, treatment of wood powder with 1% (v/v) H_2O_2 in tap water for a period of 3-5 hours at pH 11.5 was found sufficient to oxidize lignin to the point of extinction. In the practical sense the methodology standardized was simple and comparatively less expensive. A few attempts have been made by earlier workers in immobilizing nitrifiers on natural polymeric substances. In such studies wood chips and wheat straw had given comparable denitrification rates to commercial plastic media even though structural transformation and physical degradation have been observed. A commercial carrier material named 'chitopearl' is available for immobilization (www.fujibo.co.jp) but its commercial viability is questionable. The mode of immobilization to be achieved on wood powder and chitin, as the substrata, is adsorption, which has been proved as the best method of choice for immobilizing nitrifying bacteria. Entrapment in hydrogels (Vogelsang *et al.* 2002) or immobilization on carrageenan (Wijffels and Tramper, 1989) caused oxygen consumption inside the gel matrix due to increase in cell density and growth, overriding rate of oxygen diffusion across the gel matrix. This may manifest as oxygen deficiency in the core of the gel matrix (Catalan-Sakairi *et al.* 1997) resulting in poor nitrification rates.

Based on the above previous experiences by several workers, it was decided to screen and select the most appropriate substratum from among the five categories of the materials short listed for the development of the technology. The first round of screening of the substratum was on the basis of surface profile and accordingly delignified *A. altissima* (300-500μm and 500-710μm) particles exhibited the largest number of corrugations indicating more surface area for attachment. In the second round of screening, two dimensional surface areas of the samples by image analysis of scanning electron micrographs was accomplished and the particles of *A. altissima* was found to have the highest surface area. Through BET analysis specific surface area of the substratum was determined as 1.87m^2/g. This value was used for all further calculations. BET analysis has already been recognized as the standard procedure for determining surface area of highly porous substrata. Shan and Obbard (2001) using the same method determined the surface area of sterile buoyant porous clay pellet to have 1.3 to 3.4 m^2/g. Based on these estimations, particles of *A. altissima* are comparable to the clay particles to give extensive

surface area for immobilization. Being biodegradable, after a period of time, the substratum may get disintegrated leaving no residue.

It was essential to confirm the primary selection made here of the substratum by further rounds of screening based on preference of the NBC in terms of adsorption on to the particles and their nitrification potential. To facilitate the above, activation of the NBC stored at 4°C could be accomplished by inoculating and incubating 10mL consortia in Watson's medium (1L) under aeration in obscurity. For all practical purposes enumeration of cells in NBC was a challenge, which was addressed by ultrasonication at 125W for 4 minutes. Application of ultrasonication for de-aggregation of bacterial floc and biofilm has been proved successful for enumeration by Salhani and Deffur (1998).

On experimenting with the dissociated cells in all the five category of the substrata *A. altissima* with particle size 300-500 and 500-710μm was found to support uniformly all NBC in terms of least coefficient of variance (Cv). Subsequently by way of preferential attachment of the NBC the substratum derived from *A. altissima* with particle size 300-1500μm was identified and confirmed for all further immobilization purposes.

Time course of immobilization of NBC was investigated and within 24 hours itself biofilm formation was found much progressed under optimum conditions. Prolonged incubation for immobilization was leading to disintegration of the substratum. Shan and Obbard (2001) on experimenting with nitrifying bacteria immobilized on clay pellets experienced a lag phase of two to four days prior to the initiation of TAN removal in primary and subcultures. In this context formation of biofilm within 24 hours on the wood powder by all NBC suggest the suitability of the substratum for immobilization by adsorption.

Experiments to determine the least number of cells required for immobilizing in unit weight / area of the substratum suggested that 10^5 to 10^8 cells per mL in 100mL medium did not make any significant differences for immobilization on 1g substratum. This led to the realization that the lowest count of 10^5 cells per mL would be sufficient to get the cells attached satisfactory in 1g substratum per 100mL medium. This suggest that even though the cell count during immobilization happens to be lower comparatively, on adsorption and colonization they can grow rapidly forming biofilm and perform nitrification at the same rate of the immobilized substratum with initial higher number of bacterial cells.

For economic viability minimum quantity of the immobilized NBC required for treating unit volume of sea water was determined and found that within the range 0.2 to

1.0g immobilized NBC there was progressive increase of nitrification in 20L Seawater at 15 and 30ppt salinity suggesting that the quantity of immobilized NBC was directly correlated with activity. However, the nitrification per se with in the range tested was not significantly different. This offered much flexibility to the choice in the quantity of the immobilized NBC for application. Similar observations have been made by Shan and Obbard (2001) on experimenting with clay pellets immobilized with enriched cultures of nitrifiers at low pellet dosage of 1pellet (1g, surface area 1.3 to 3.4m^2/g) for 4L of pond water, a lag phase of TAN removal was not apparent. Mean while in the present experiment comparable results could be obtained with 0.2 g (surface area 1.87m2/g) substratum immobilized with NBC in 20L seawater at salinities 15 and 20. This is a clear demonstration of better quality of immobilized nitrifiers generated in this study for field application.

In all commercial applications of the immobilized NBC shelf life happens to be a major issue to be sorted out. In this case storage of the immobilized nitrifiers for a month under ambient conditions did not affect much the nitrifying potency; however, TAN removal rates were slightly reduced. This is an after effect of long term storage which has generally been experienced both with pure cultures and consortia. But when they are brought to a fresh medium and incubated under aeration and agitation the activity have been found regaining. This suggests that before application, the immobilized nitrifiers have to be aerated and agitated to perform better.

Surprisingly the NBC were found with denitrification potency. On examining the same under anaerobic conditions, NO_3-N removal was well correlated with NO_2-N suggesting the existence of dissimilatory denitrification. This observation was quiet true in the sense that the pure cultures resolved from the consortia were with denitrifying potency. This situation is advantageous in the sense that a single product on application can perform both nitrification and denitrification.

On evaluating the nitrifying potency of immobilized NBC in a simulated bioassay system the extend of nitrification was double the one recorded in control suggesting their probable efficacy under field conditions at salinities 10, 20 and 32. This experiment proves the usefulness of the product on mitigating ammonia toxicity in aquaculture systems.

Subsequently, a simple device was designed and fabricated for mass immobilization of NBC. A three day treatment in this system was found satisfactory to effectively immobilize the nitrifiers for field application. Further incubation did not add to the efficacy. The device is simple and user friendly and can be manufactured and operated

with less capital investment and operational cost. However, more studies have to be conducted on the kinetics of immobilization and the shelf life of the product generated from this system. A point of concern is the involvement and interference of natural microbial flora present in the seawater used for suspending the bacterial consortium and the substratum for immobilization. According to the protocol which has been experimented the sea water was not sterilized by any means. An extensive and thorough analysis of this situation is most warranted.

One of the consortia (AMOPCU-1) on applying in a shrimp grow out system, 50% lowering of TAN was recorded. Even though from this single experiment it may not be possible to conclude the efficacy of the immobilized NBC in bioremediating ammonia toxicity in shrimp grow out systems. The data generated suggest that by repeated trial and error method an efficient bioremediation package for ammonia toxicity for shrimp grow out systems can be brought out with the products which have been generated in this study. The focus of future research must be on standardization of mass immobilization of nitrifiers on wood powder substratum and development of an appropriate application module for instantaneous and sustainable ammonia removal.

Fig. 3.1: Schematic representation of mass immobilization device.

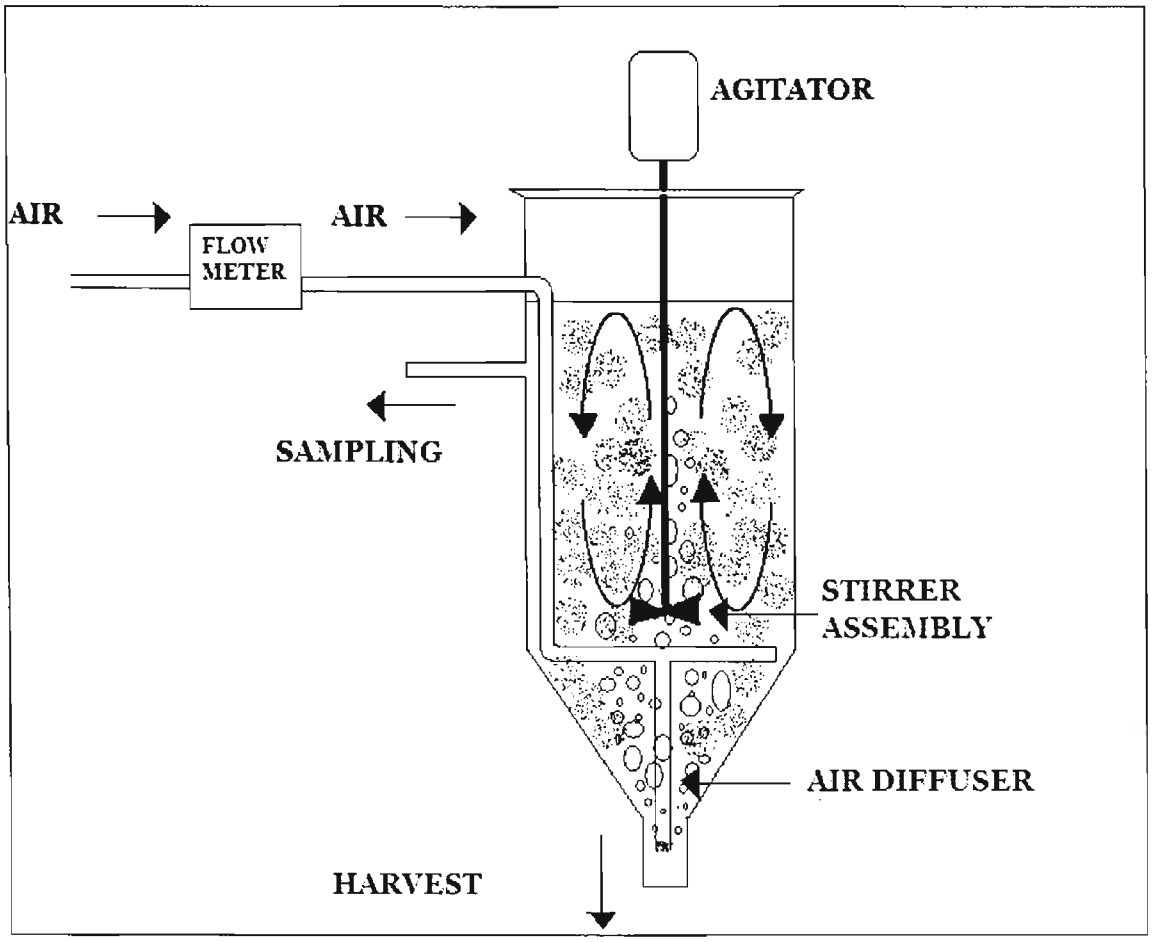


Table 3.1: Efficacy of delignification protocols.

Sl. No.	Composition of reaction mixture	Duration (Hour)	Plant species and particle size	Lignin *
1	0.2% (v/v) H_2O_2 in DW pH 11.5	24	*Ailanthus altissima* (300-1500µm)	Present
2	0.4% (v/v) H_2O_2 in DW pH 11.5	24		Present
3	0.6% (v/v) H_2O_2 in DW pH 11.5	24		Present
4	0.8% (v/v) H_2O_2 in DW pH 11.5	24		Present
5	1.0% (v/v) H_2O_2 in DW pH 11.5	24		Absent
6	1.5% (v/v) H_2O_2 in DW pH 11.5	24		Absent
7	2.0% (v/v) H_2O_2 in DW pH 11.5	24		Absent
8	1.0% (v/v) H_2O_2 in DW pH 11.5	2		Present
9	1.0% (v/v) H_2O_2 in DW pH 11.5	3		Absent
10	1.0% (v/v) H_2O_2 in DW pH 11.5	4		Absent
11	1.0% (v/v) H_2O_2 in DW pH 11.5	5		Absent
12	1.0% (v/v) H_2O_2 in Tap water pH 11.5	3		Absent

* Presence of lignin was indicated by the magenta coloured liquor subsequent to chlorination and addition of aqueous hot sodium sulphite.

Fig. 3.2: Appearance of delignified wood powder.

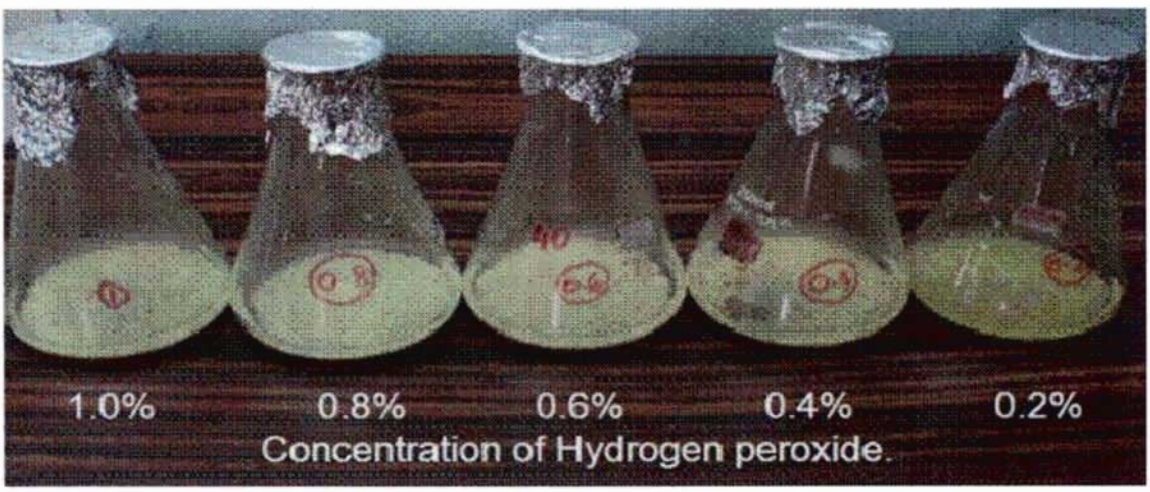
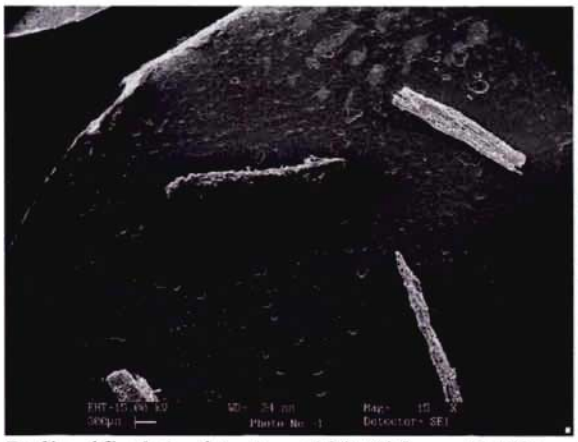
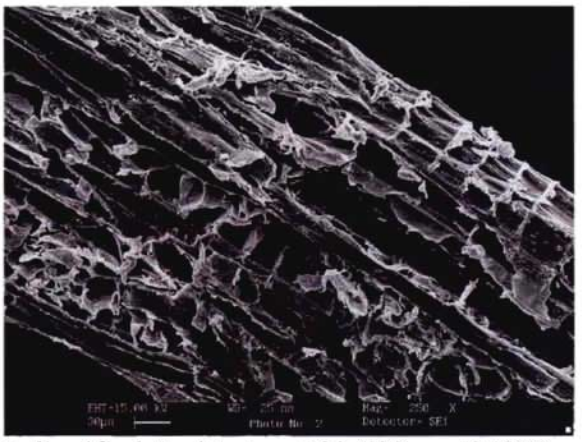


Fig. 3.3: Scanning electron micrographs of different carrier materials.

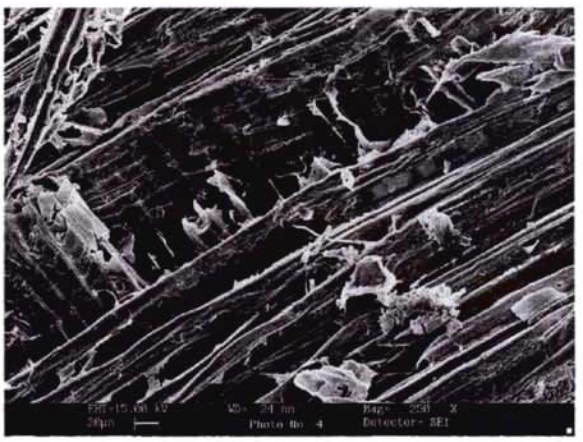


Delignified *A altissima* 300-500µm (15X)

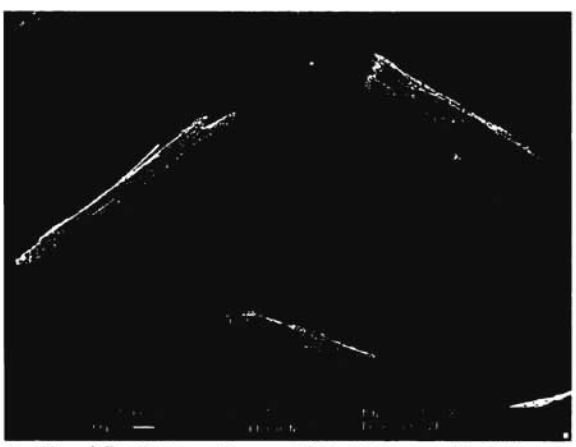


Delignified *A altissima* 300-500µm (250X)

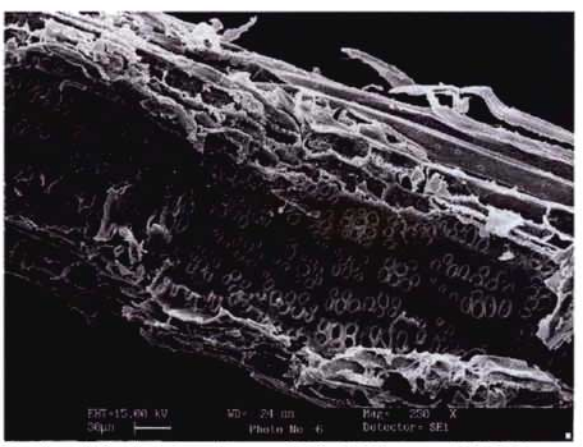
Delignified *A altissima* 500-700µm (15X)



Delignified *A altissima* 500-700µm (250X)



Delignified *M peltata* 300-500 µm (15X)



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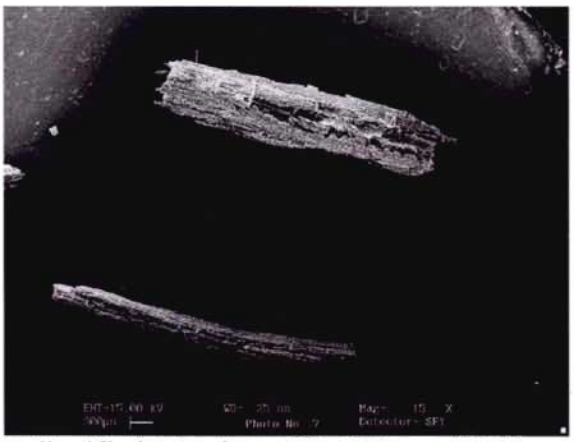
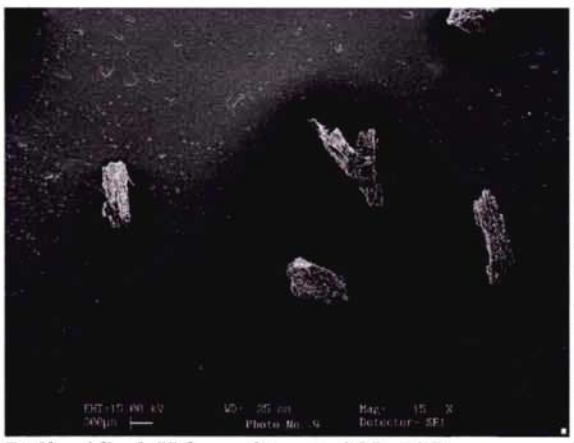
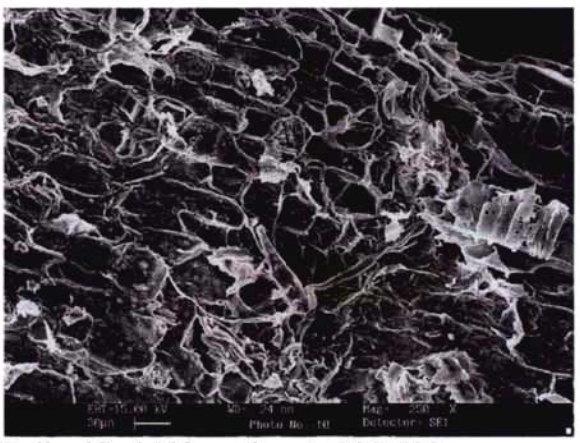


Delignified *M peltata* 500-700 μm (15X)

Delignified *M peltata* 500-700 μm (250X)



Delignified *H braziliensis* 300-500 μm (15X)



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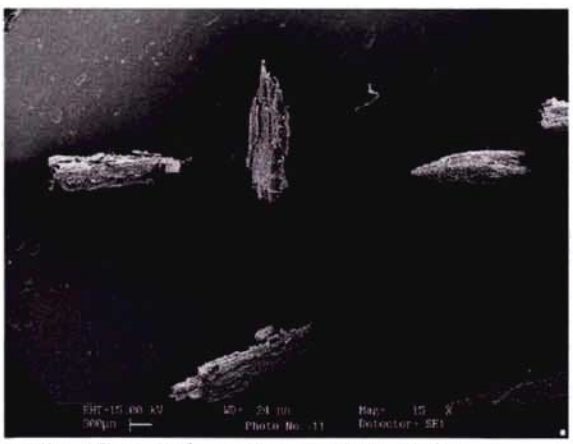
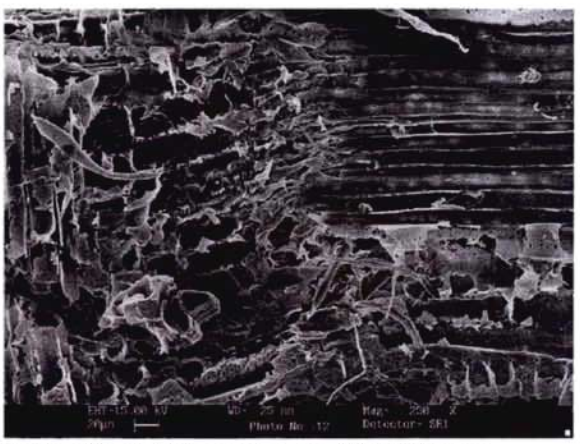
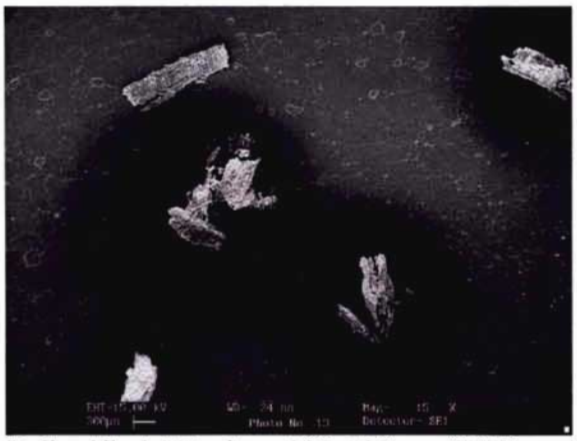


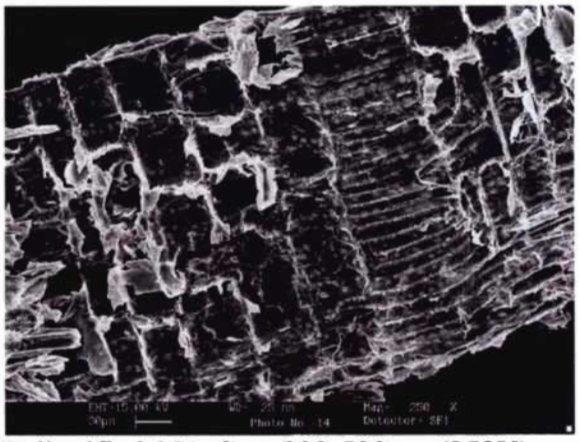
Delignified *H braziliensis* 500-700 μm (15X)



Delignified *H braziliensis* 500-700 μm (250X)



Delignified *M indica*, 300-500µm (15X)



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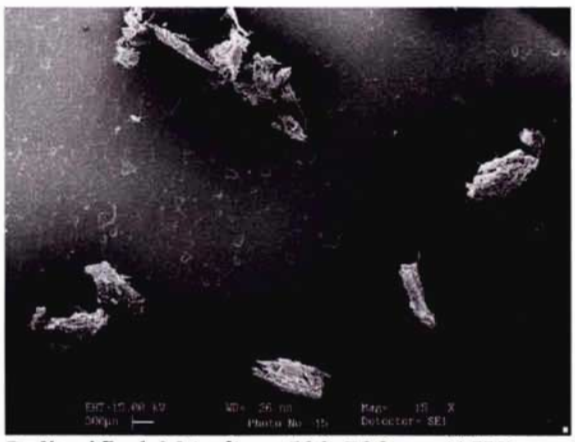
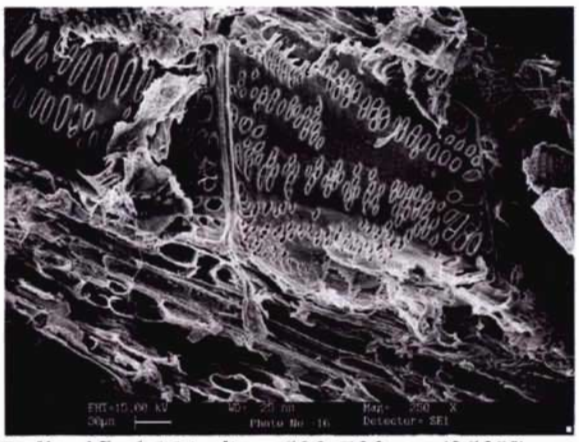
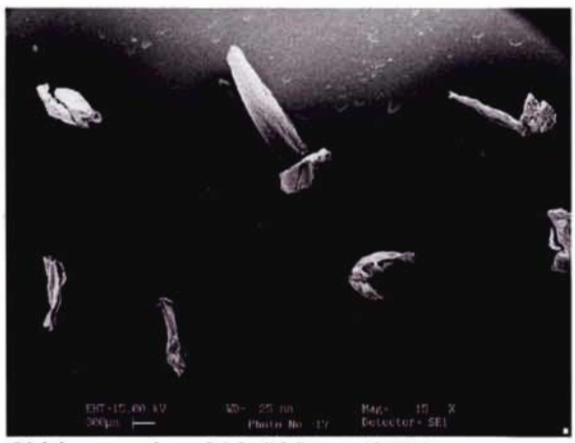


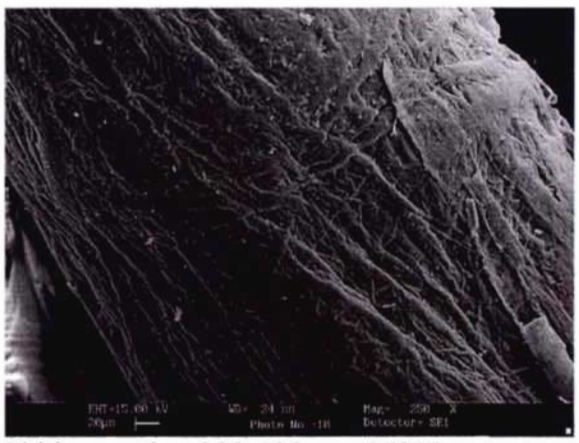
Delignified *M indica*, 500-700µm (15X)



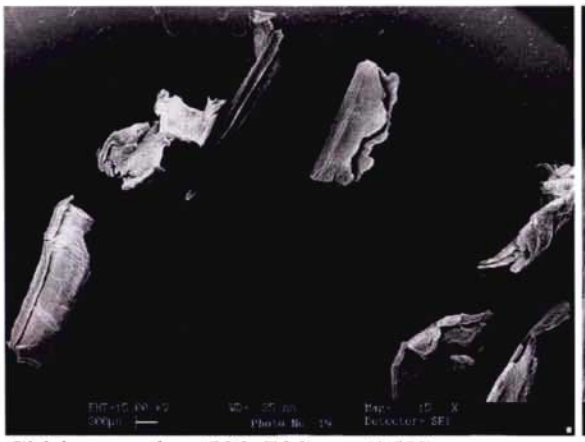
Delignified *M indica*, 500-700µm (250X)



Chitin powder, 300-500µm (15X)



Chitin powder, 300-500µm (250X)



Chitin powder, 500-700μm (15X)

Chitin powder, 500-700μm (250X)

Table 3.2: Two dimensional image analysis of the substrata.

No.	Particles.	2D area* (m^2/g)
1	*Ailanthus altissima* (300-500μm)	0.70
2	*Ailanthus altissima* (500-710μm)	0.60
3	*Macaranga peltata* (300-500μm)	0.60
4	*Macaranga peltata* (500-710μm)	0.40
5	*Hevea braziliensis* (300-500μm)	0.44
6	*Hevea braziliensis* (500-710μm)	0.30
7	*Mangifera indica* (300-500μm)	0.56
8	*Mangifera indica* (500-710μm)	0.27
9	Chitin (300-500μm)	0.36
10	Chitin (500-710μm)	0.30

* based on 2D image analysis.

Fig. 3.4: Cell count at ultrasonication of the nitrifying bacterial consortia.

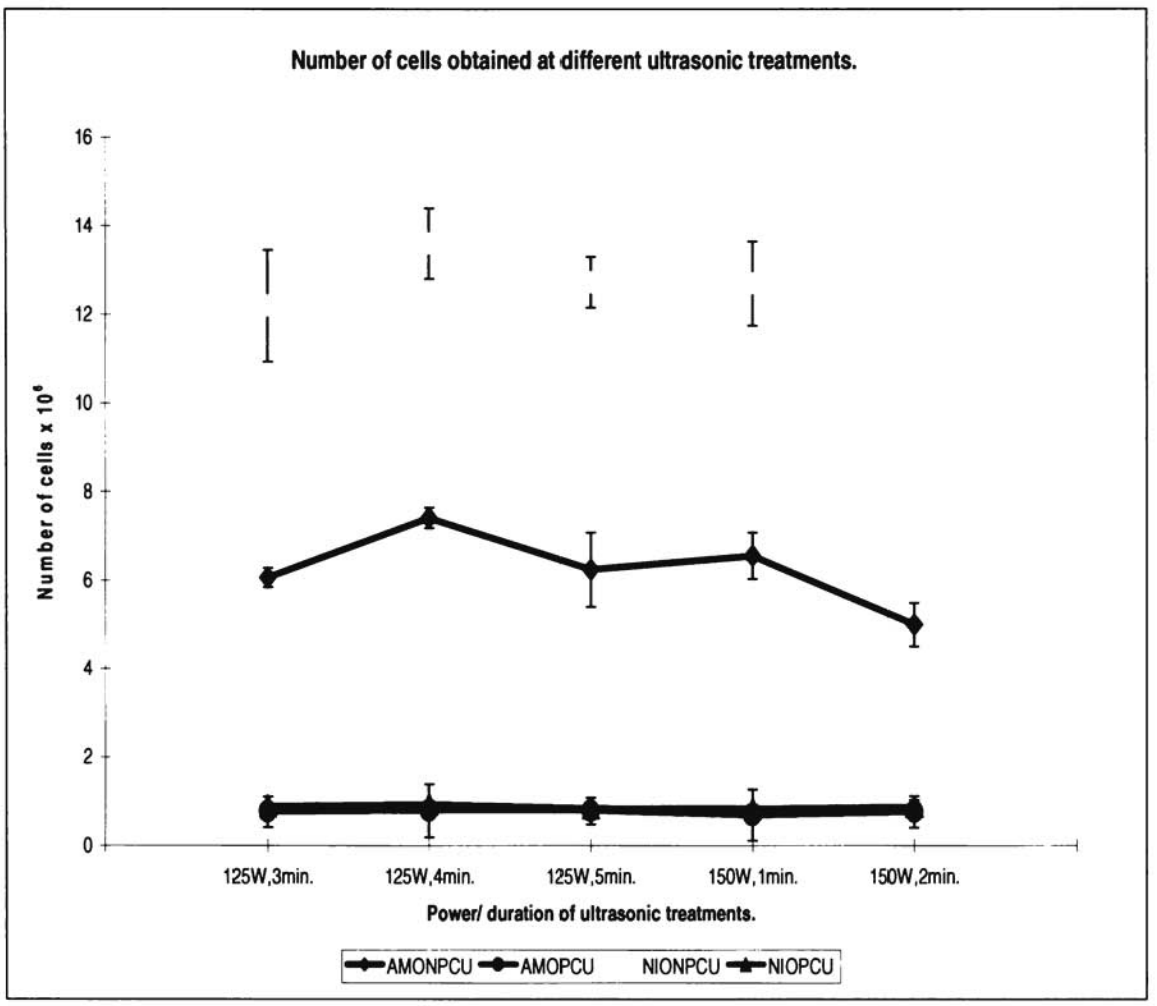


Table 3.3: Total TAN removal in mg/L recorded during the 10 day incubation of the nitrifying bacterial consortia with the substrata.

Carrier materials used	Nitrifying bacterial consortia.				Coeft.of Variance
	AMONPCU-1	AMOPCU-1	NIONPCU-1	NIOPCU-1	
Ailanthus altissima (300-500µm)	42	58	72	63	18.50%
Ailanthus altissima (500-710µm)	63	64	63	74	7.03%
Macaranga peltata (300-500µm)	47	63	69	66	13.88%
Macaranga peltata (500-710µm)	61	64	73	73	7.90%
Hevea braziliensis (300-500µm)	54	60	72	42	18.90%
Hevea braziliensis (500-710µm)	37	49	66	66	22.60%
Mangifera indica (300-500µm)	52	61	72	74	13.70%
Mangifera indica (500-710µm)	54	63	76	77	14.10%
Chitin (300-500µm)	68	30	76	76	30.40%
Chitin (500-710µm)	75	47	70	77	17.80%

Table 3.4: Total TAN removal in mg/L recorded for 4 days after transferring the substrata with immobilized nitrifiers to fresh medium.

Carrier materials used	Nitrifying bacterial consortia				Coeft.of Variance
	AMONPCU-1	AMOPCU-1	NIONPCU-1	NIOPCU-1	
Ailanthus altissima (300-500µm)	14.0	7.7	16.3	10.8	26.60%
Ailanthus altissima (500-710µm)	14.1	14.5	15.9	13.8	5.80%
Macaranga peltata (300-500µm)	13.5	13.0	8.9	10.5	15.70%
Macaranga peltata (500-710µm)	16.5	16.5	10.8	12.2	18.20%
Hevea braziliensis (300-500µm)	9.8	10.2	16.9	16.5	25.20%
Hevea braziliensis (500-710µm)	13.3	16.7	17.5	15.6	8.23%
Mangifera indica (300-500µm)	12.8	9.2	16.7	14.3	20.60%
Mangifera indica (500-710µm)	16.5	16.2	17.7	17.7	3.82%
Chitin (300-500µm)	10.4	16.5	14.3	14.3	15.90%
Chitin (500-710µm)	9.5	17	16.8	15.9	20.90%

Fig. 3.5: Effect of particle size of the substratum (wood powder from *A. altissima*) on TAN removal on immobilizing the nitrifying bacterial consortia.

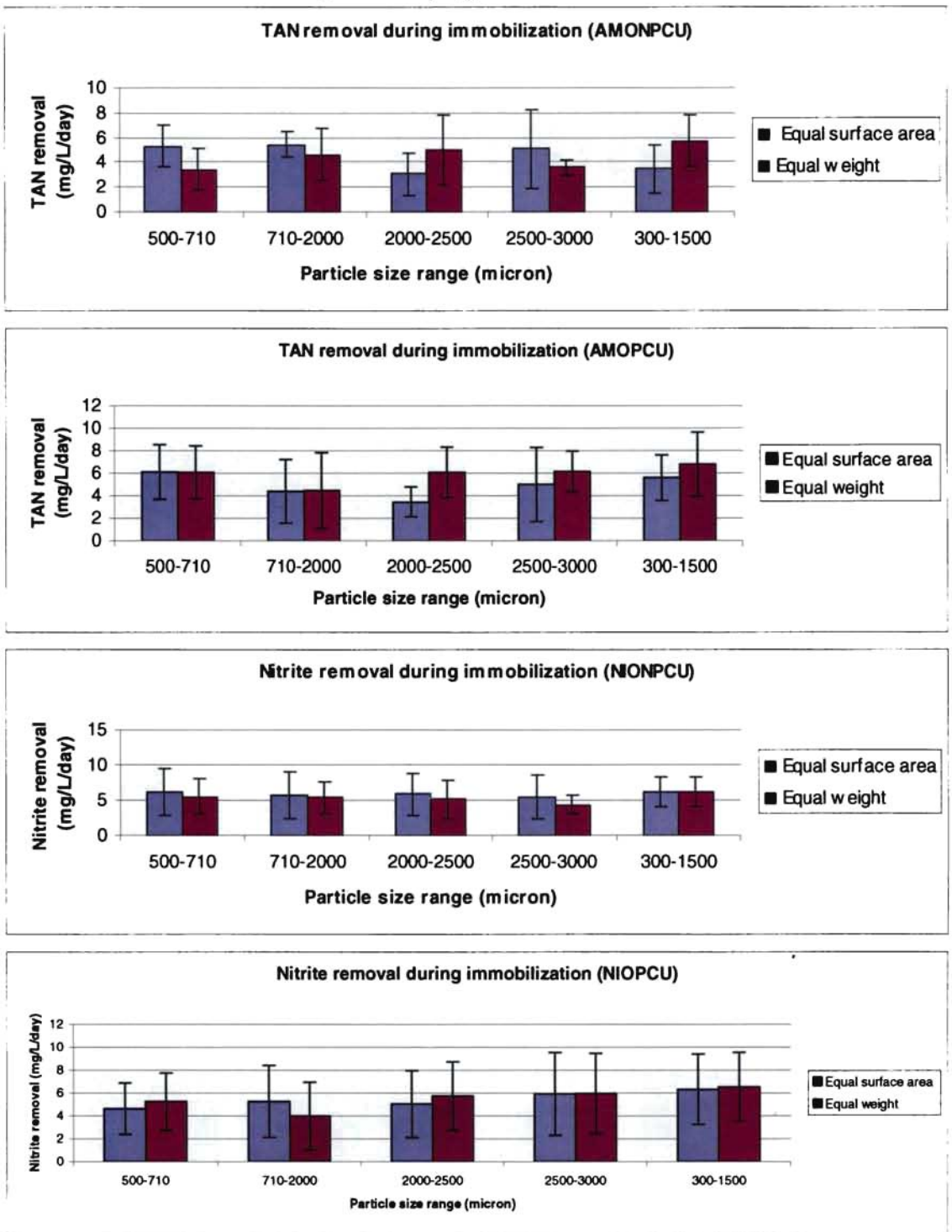


Fig. 3.6: Effect of particle size of wood powder on TAN removal in fresh medium.

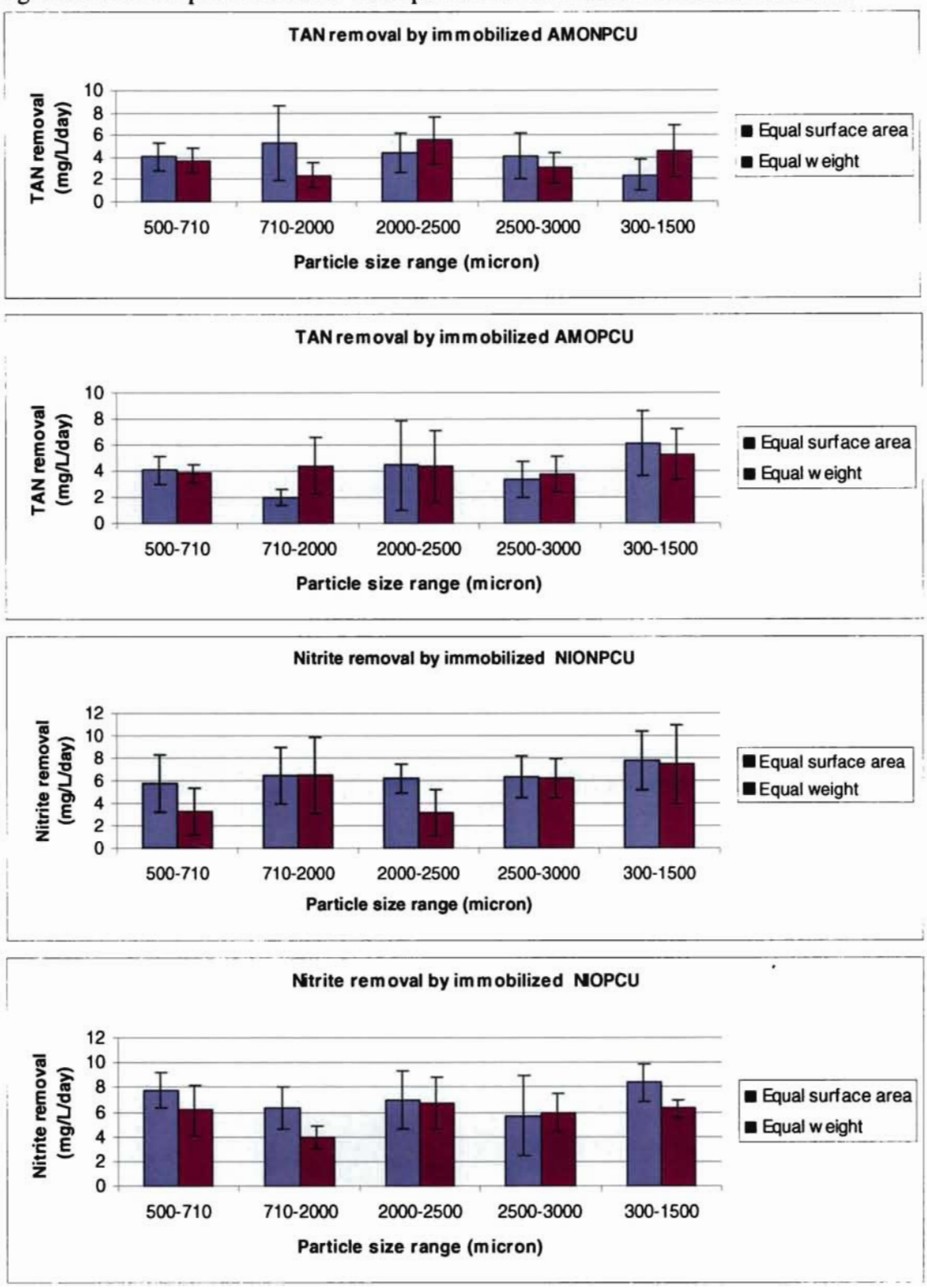
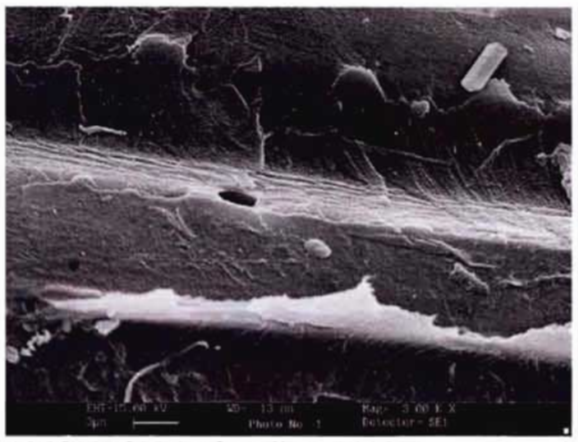
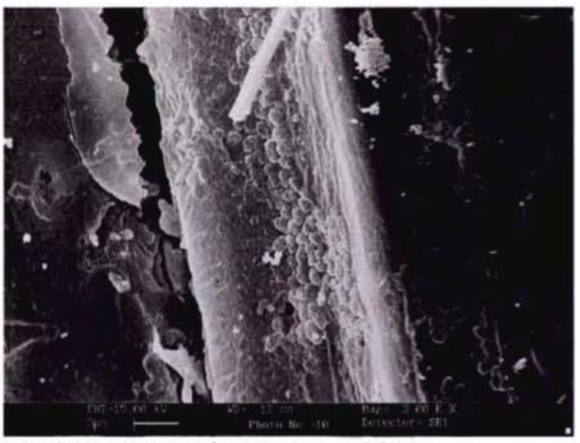


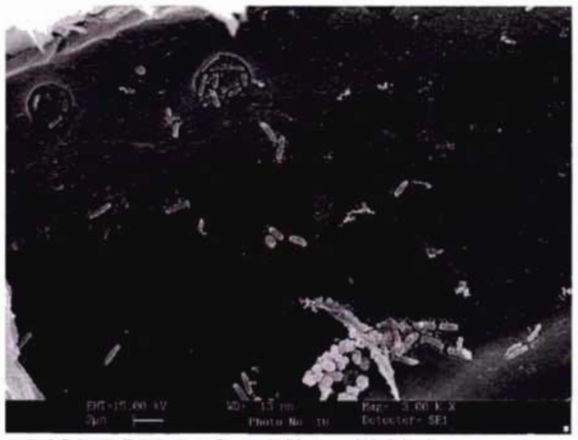
Fig. 3.7: Biofilm development stages during immobilization.



AMONPCU-1 after 6hrs, 3000X

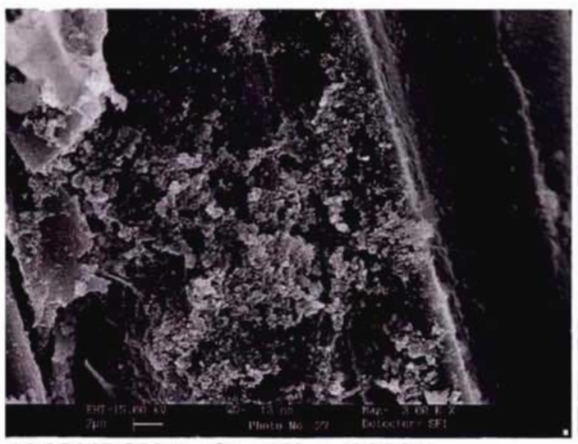


AMONPCU-1 after 12hrs, 3000X

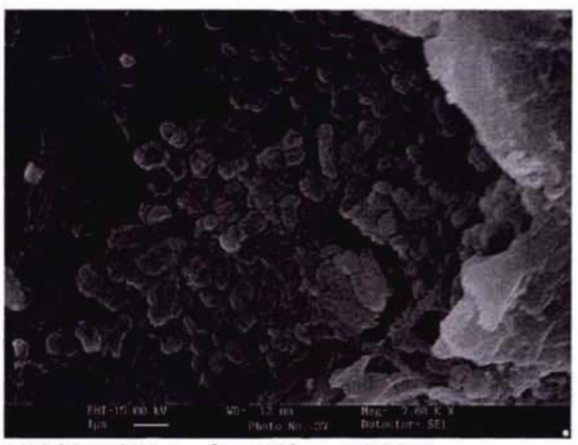


AMONPCU-1 after 24hrs, 3000X

AMONPCU-1 after 24hrs, 7000X



AMONPCU-1 after 48hrs, 3000X

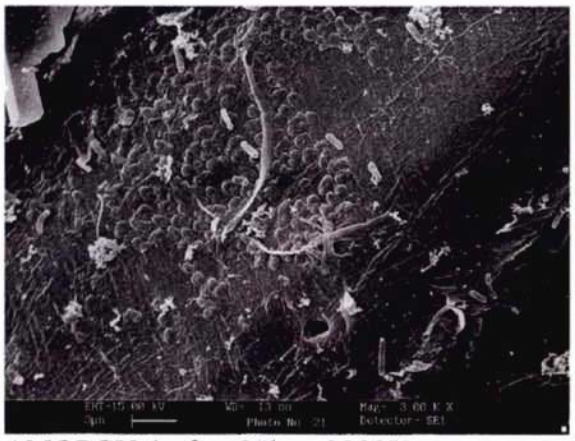


AMONPCU-1 after 72hrs, 7000X

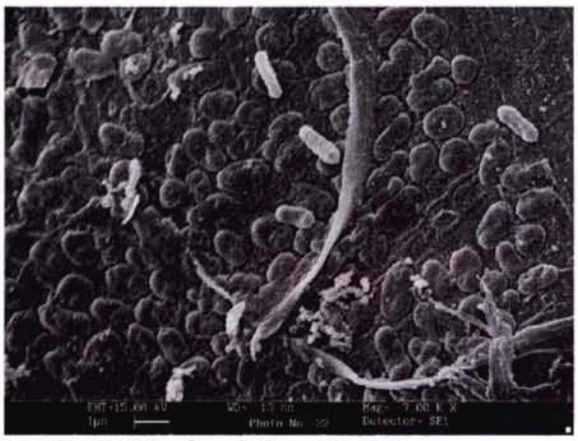
Fig. 3.8: Biofilm development stages of AMOPCU-1

AMOPCU-1 after 6hrs, 3000X

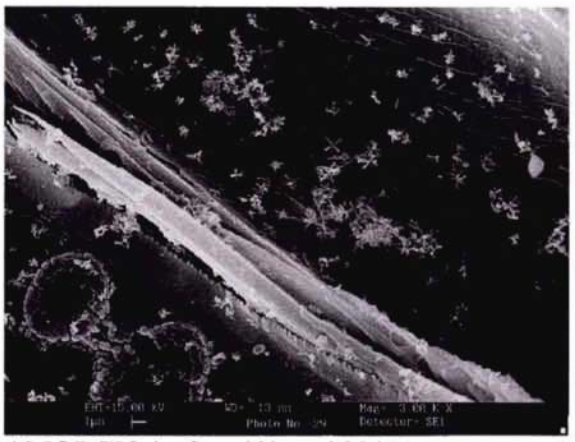
AMOPCU-1 after 6hrs, 3000X



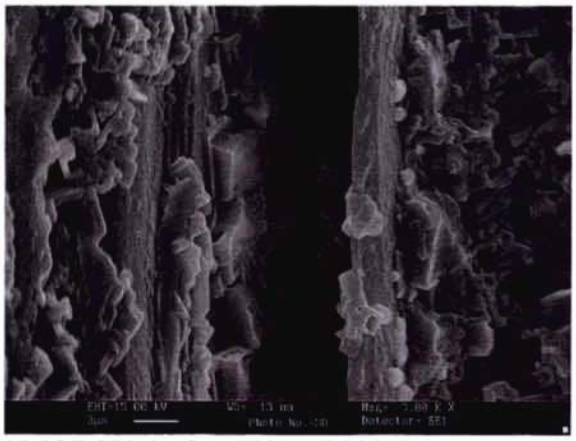
AMOPCU-1 after 24hrs, 3000X



AMOPCU-1 after 24hrs, 7000X

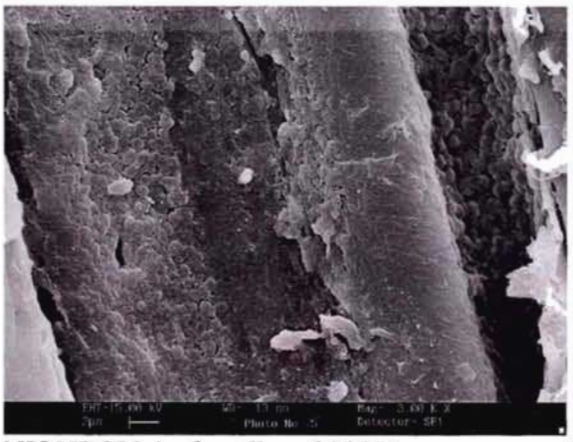


AMOPCU-1 after 48hrs, 3000X

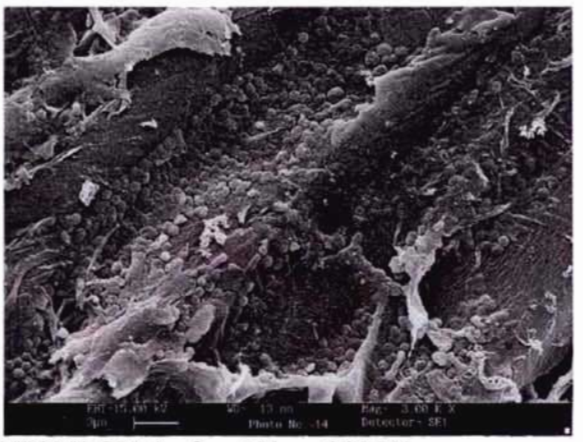


AMOPCU-1 after 72hrs, 3000X

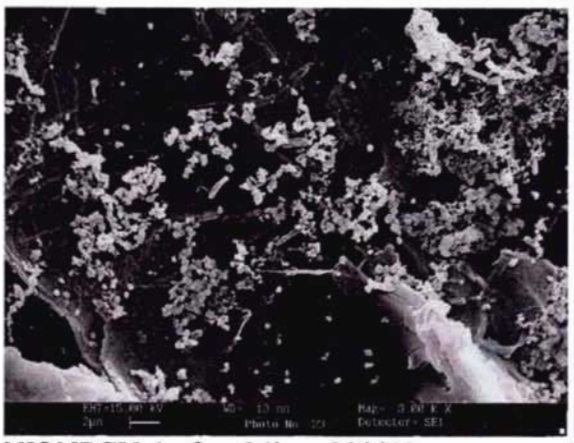
Fig. 3.9: Biofilm development stages of NIONPCU-1



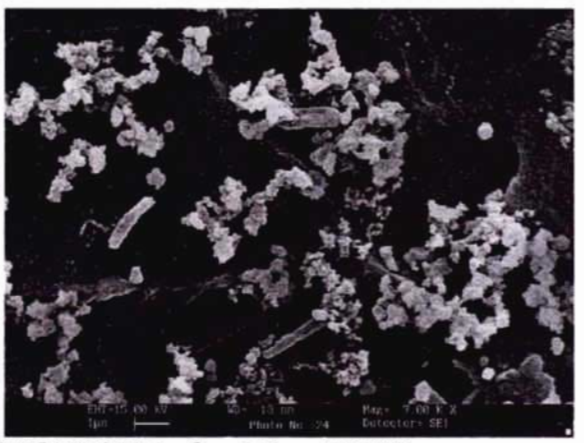
NIONPCU-1 after 6hrs, 3000X



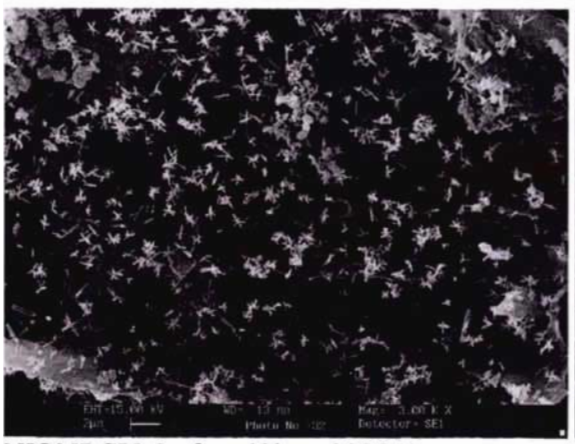
NIONPCU-1 after 12hrs, 3000X



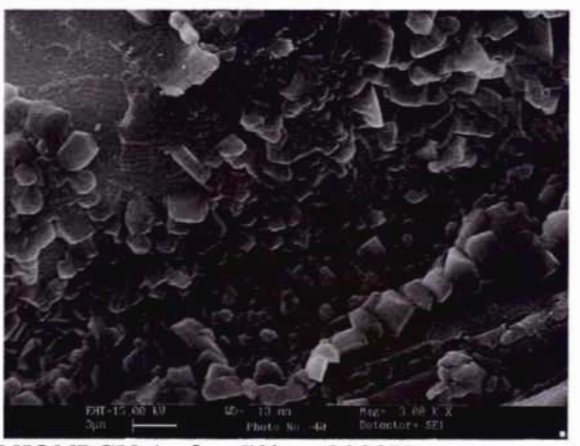
NIONPCU-1 after 24hrs, 3000X



NIONPCU-1 after 24hrs, 7000X

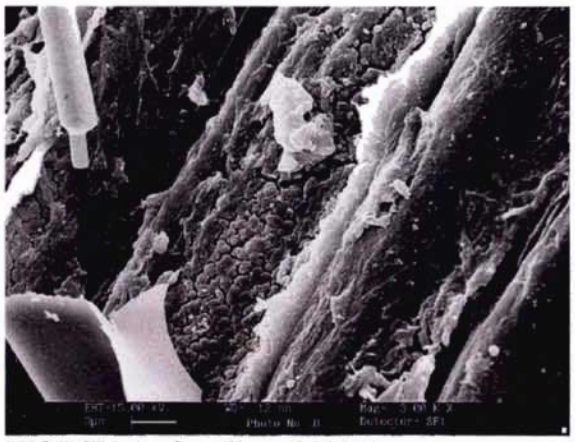


NIONPCU-1 after 48hrs, 3000X

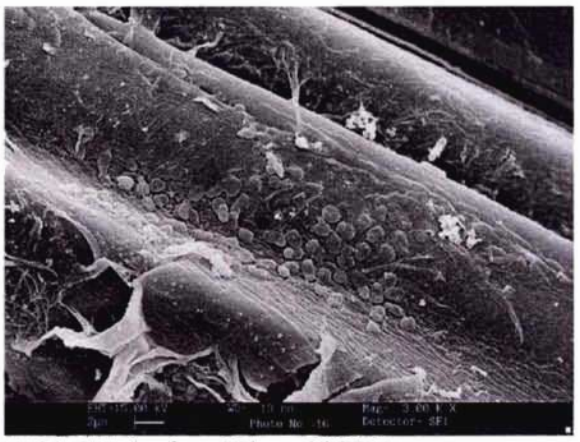


NIONPCU-1 after 72hrs, 3000X

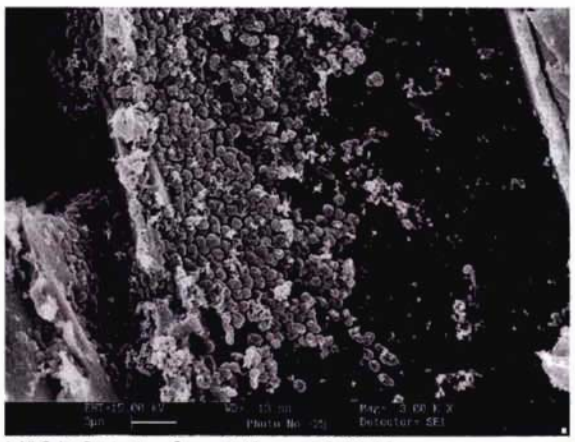
Fig. 3.10: Biofilm development stages of NIOPCU-1



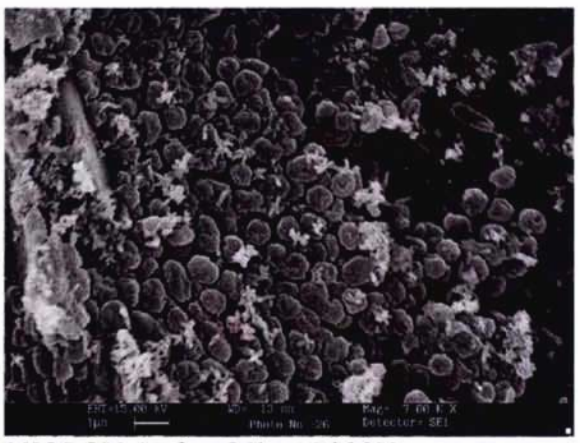
NIOPCU-1 after 6hrs, 3000X



NIOPCU-1 after 12hrs, 3000X



NIOPCU-1 after 24hrs, 3000X



NIOPCU-1 after 24hrs, 7000X

NIOPCU-1 after 48hrs, 7000X

NIOPCU-1 after 72hrs, 7000X

Fig. 3.11: Relation between initial cell density and TAN removal rate.

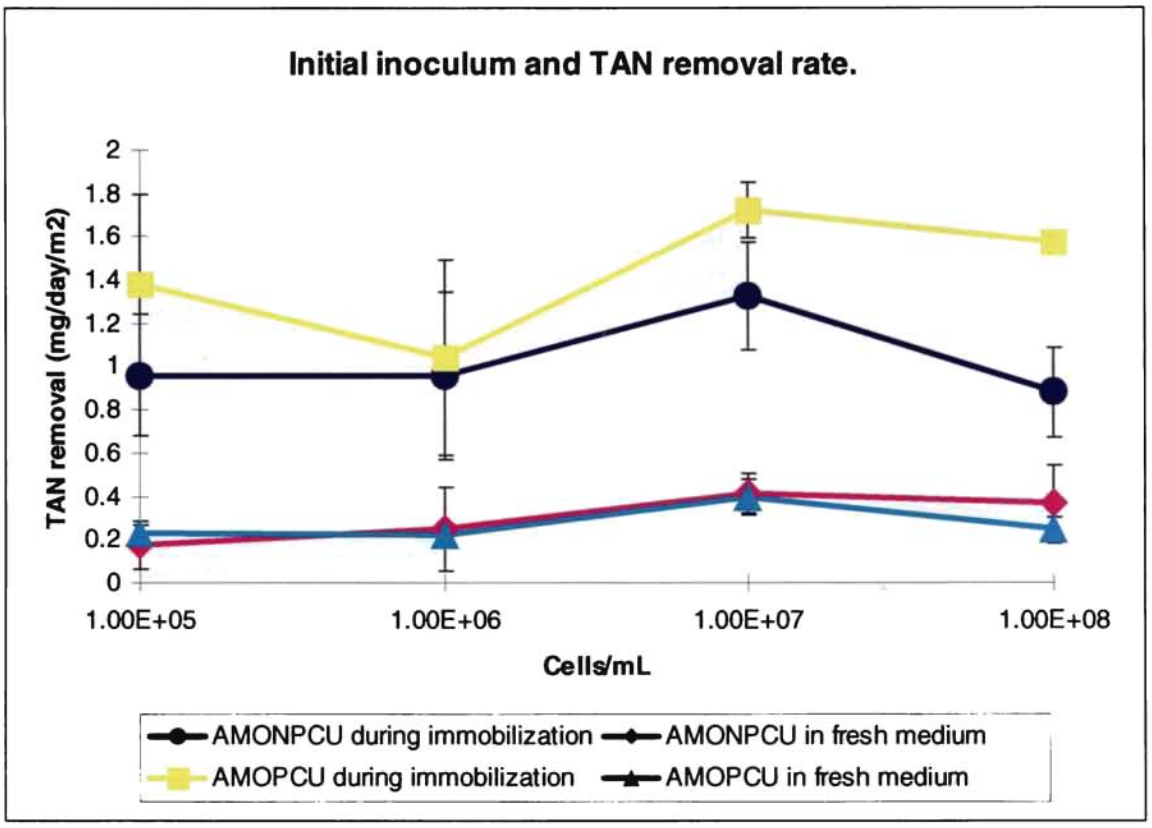


Table 3.5: Activity during immobilization of NBC used for determining the minimum quantity required for the treatment of unit volume of sea water.

Consortia		AMONPCU	AMOPCU
TAN removal mg/L/Day	Day1	5.34 ± 0.323	5.86 ± 0.462
	Day2	7.01 ± 0.223	7.86 ± 0.433
	Day3	6.67 ± 1.050	6.58 ± 0.644
	Day4	6.84 ± 0.458	5.09 ± 0.505

Table 3.6: Minimum quantity of immobilized wood powder required for treating unit volume of sea water.

Quantity of immobilized substrata	Average TAN removal		
	Day 1*	Day 2**	Day 3***
AMONPCU 0.2g/ 20L sea water (15ppt)	1.29±0.21	0.52±0.12	0.61±0.08
AMONPCU 0.4g/ 20L sea water (15ppt)	1.11±0.09	0.62±0.16	0.76±0.04
AMONPCU 0.6g/ 20L sea water (15ppt)	1.15±0.13	0.72±0.1	0.64±0.01
AMONPCU 0.8g/ 20L sea water (15ppt)	0.85±0.23	0.91±0.32	0.96±0.06
AMONPCU 1.0g/ 20L sea water (15ppt)	0.62±0.12	0.63±0.11	1.2±0.08
20L sea water (15ppt) as control	0.35±0.03	0.4±0.1	0.19±0.002
AMOPCU 0.2g/ 20L sea water (30ppt)	0.65±0.12	0.63±0.21	0.66±0.02
AMOPCU 0.4g/ 20L sea water (30ppt)	0.79±0.1	0.64±0.08	0.95±0.07
AMOPCU 0.6g/ 20L sea water (30ppt)	1.04±0.02	0.82±0.1	1.21±0.1
AMOPCU 0.8g/ 20L sea water (30ppt)	0.93±0.12	0.74±0.14	1.2±0.08
AMOPCU 1.0g/ 20L sea water (30ppt)	1.05±0.08	0.87±0.26	1.03±0.003
20L sea water (30ppt) as control	0.57±0.08	0.4±0.2	0.19±0.05

r between quantity and TAN removal in 15ppt * 0.01044, ** 0.74304, *** 0.93152.
r between quantity and TAN removal in 30ppt * 0.92427, ** 0.90799, *** 0.83198.
$p = 0.162565$ (15ppt), $p = 0.003312$ (30ppt)

Quantity of immobilized substrata	Average nitrite production		
	Day 1*	Day 2**	Day 3***
AMONPCU 0.2g/ 20L sea water (15ppt)	0.044±0.0003	0.006±0.004	0.036±0.009
AMONPCU 0.4g/ 20L sea water (15ppt)	0.038±0.002	0.023±0.002	0.019±0.001
AMONPCU 0.6g/ 20L sea water (15ppt)	0.061±0.01	0.01±0.004	0.017±0.007
AMONPCU 0.8g/ 20L sea water (15ppt)	0.056±0.01	0.017±0.0002	0.018±0.006
AMONPCU 1.0g/ 20L sea water (15ppt)	0.057±0.01	0.039±0.01	0.078±0.009
20L sea water (15ppt) as control	0.028±0.002	0.013±0.002	0.035±0.005
AMOPCU 0.2g/ 20L sea water (30ppt)	0.093±0.02	0.019±0	0.017±0.003
AMOPCU 0.4g/ 20L sea water (30ppt)	0.073±0.005	0.047±0.007	0.048±0.002
AMOPCU 0.6g/ 20L sea water (30ppt)	0.091±0.01	0.021±0	0.094±0.0001
AMOPCU 0.8g/ 20L sea water (30ppt)	0.088±0.002	0.034±0.004	0.049±0.003
AMOPCU 1.0g/ 20L sea water (30ppt)	0.088±0.0004	0.054±0.004	0.104±0.12
20L sea water (30ppt) as control	0.055±0.01	0.035±0.0008	0.02±0.0003

r between quantity and nitrite production in 15ppt * 0.84769, ** 0.67763, *** 0.36500.
r between quantity and nitrite production in 30ppt * 0.61063, ** 0.44022, *** 0.82215.
$p = 0.36126$ (15ppt), $p = 0.51655$ (30ppt)

Table 3.7: TAN and NO_2-N removal rate after one month storage.

Nitrifying consortia.	Aerated.		Without aeration.	
	TAN removal rate.	NO_2-N removal rate.	TAN removal rate.	NO_2-N removal rate.
Ammonia oxidizing consortia. AMONPCU-1.	0.2±0.035 mg/m^2/day.		0.05±0.02 mg/m^2/day.	
Ammonia oxidizing consortia. AMOPCU-1.	0.23±0.034 mg/m^2/day.		0.06±0.04 mg/m^2/day.	
Nitrite oxidizing consortia. NIONPCU-1.		0.18±0.07 mg/m^2/day.		0.08±0.02 mg/m^2/day.
Nitrite oxidizing consortia. NIOPCU-1.		0.16±0.05 mg/m^2/day.		0.07±0.002 mg/m^2/day.

Fig. 3.12: TAN removal before and after storage of immobilized product.

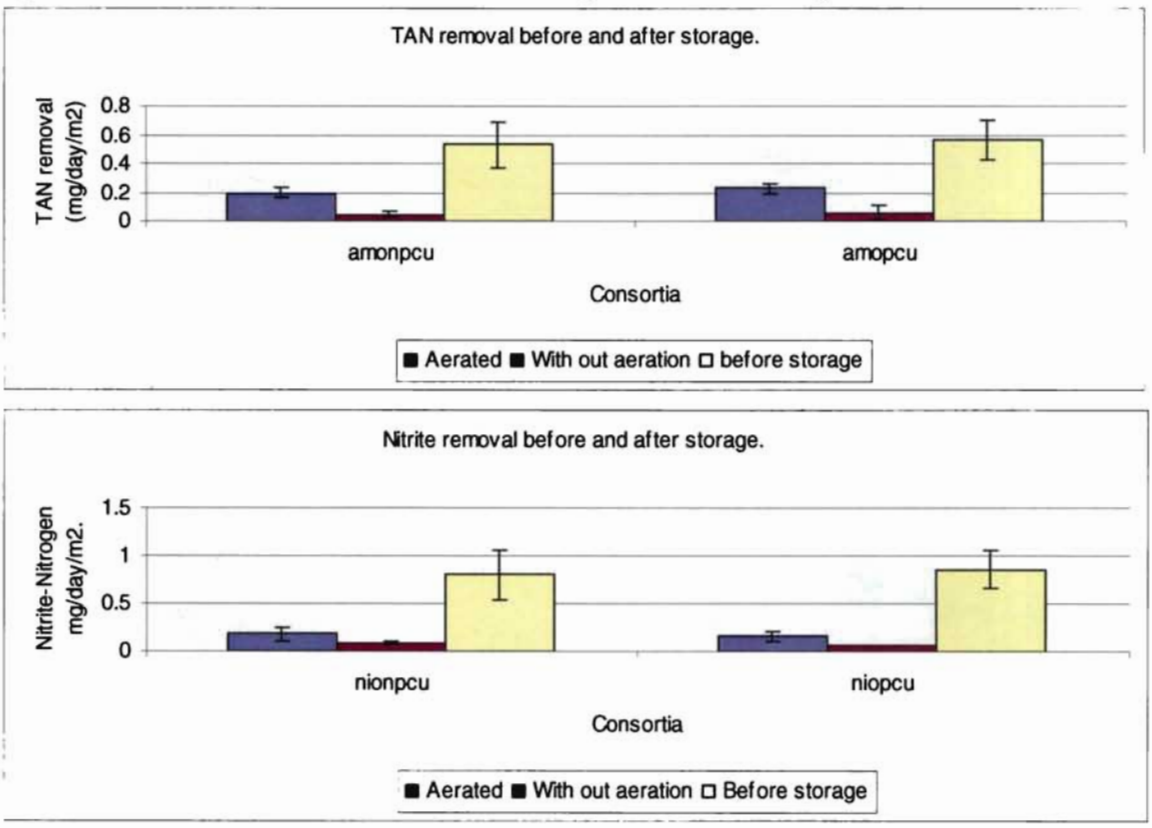


Table 3.8: Denitrification potency of the consortia

Sample specification.		NO_3-N removal rate (mg/day/10^{10} cells)	NO_2-N production rate (mg/day/10^{10} cells)	TAN production rate (mg/day/10^{10} cells)
Anaerobic condition.	AMONPCU-1	0.0600 ± 0.020	0.0007 ± 0.0000	0.0000
	AMOPCU-1	4.0033 ± 0.665	1.4550 ± 0.2758	0.0000
	NIONPCU-1	0.0470 ± 0.019	0.0245 ± 0.0035	0.0000
	NIOPCU-1	0.5553 ± 0.144	0.1855 ± 0.0205	0.0028±0.001
Aerobic condition.	AMONPCU-1	0.0604 ± 0.023	0.0070 ± 0.0035	0.0115±0.016
	AMOPCU-1	5.1333 ± 1.790	0.0223 ± 0.0186	1.3433±1.499
	NIONPCU-1	0.0547 ± 0.031	0.0127 ± 0.0070	0.0026±0.002
	NIOPCU-1	0.3063 ± 0.118	0.1202 ± 0.1904	0.0000
Medium without inoculum.	AMONPCU-1	0.0000	0.0195 ± 0.0007	0.0033±0.005
	AMOPCU-1	0.0000	0.0265 ± 0.0049	0.0000
	NIONPCU-1	0.0000	0.0390 ± 0.0057	0.0000
	NIOPCU-1	0.0012 ± 0.001	0.0670 ± 0.0114	0.0000

Fig. 3.13: Decrease in average TAN concentration in 10, 20 and 32ppt tanks.

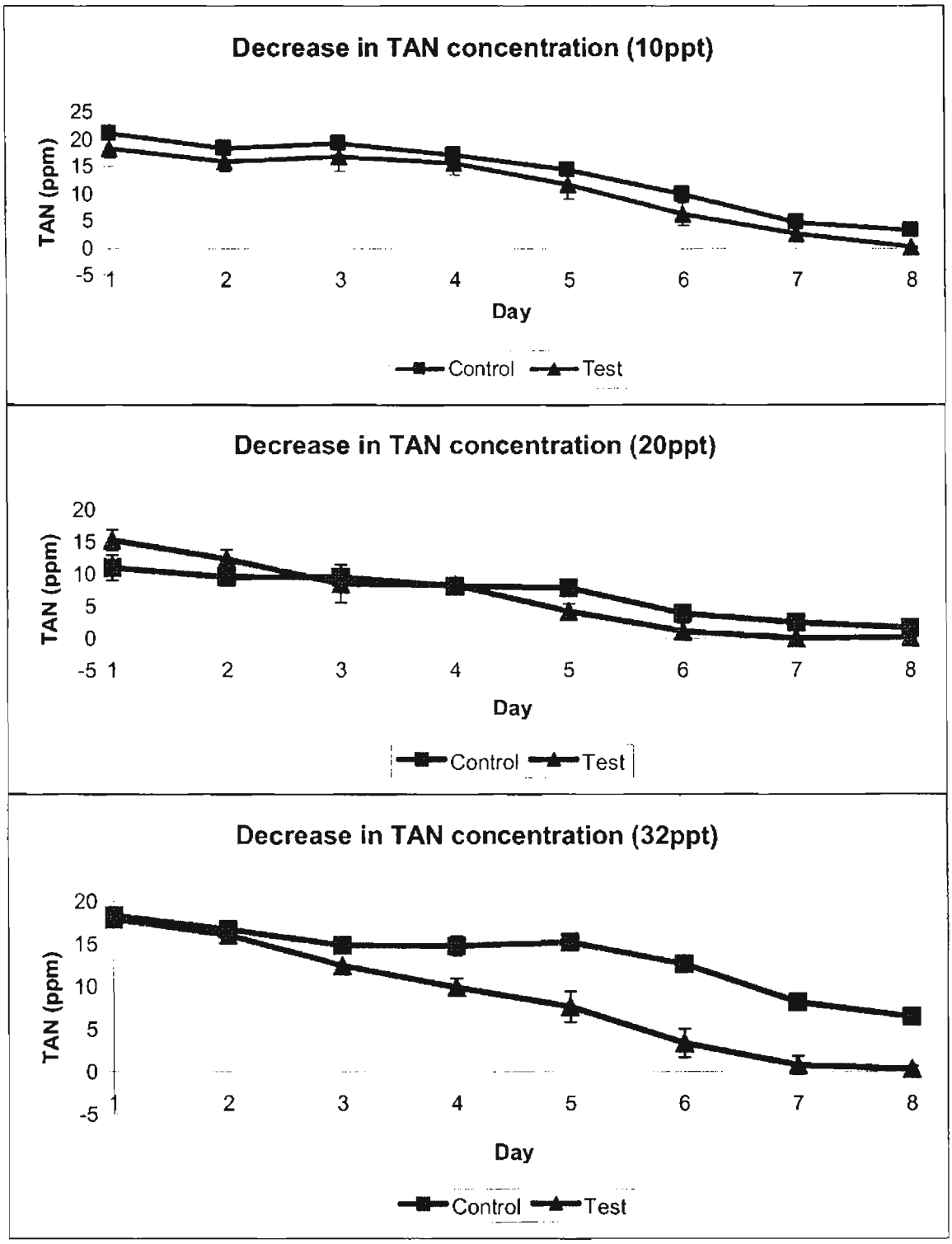
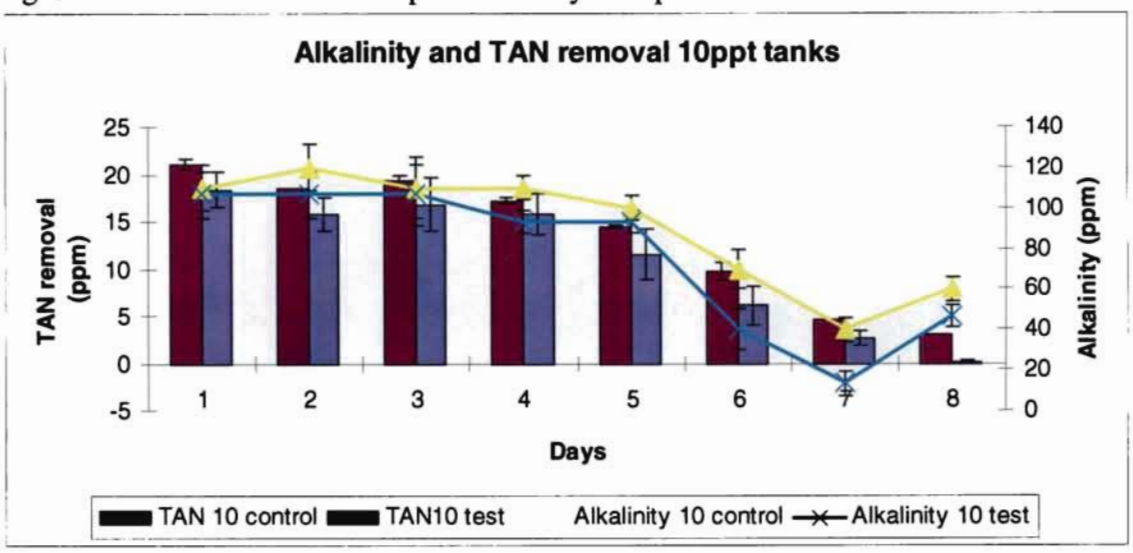
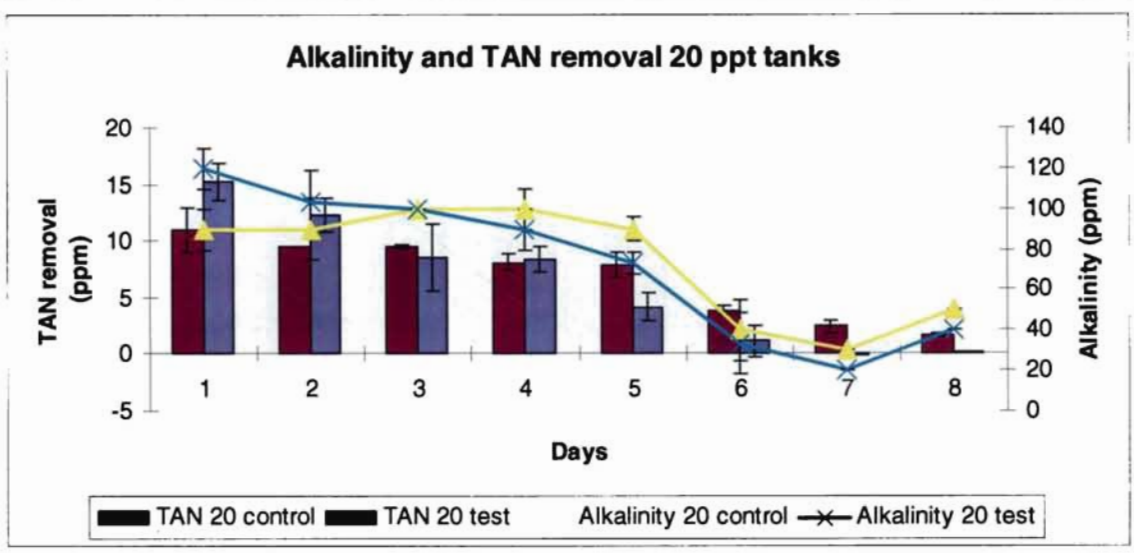


Fig. 3.14: TAN removal and drop in alkalinity in experimental tanks.





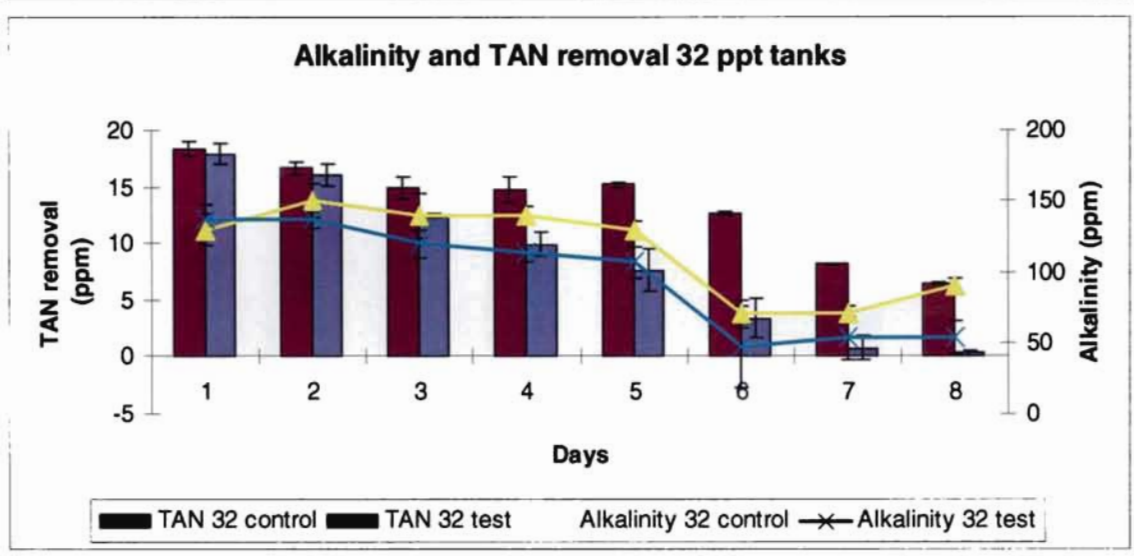
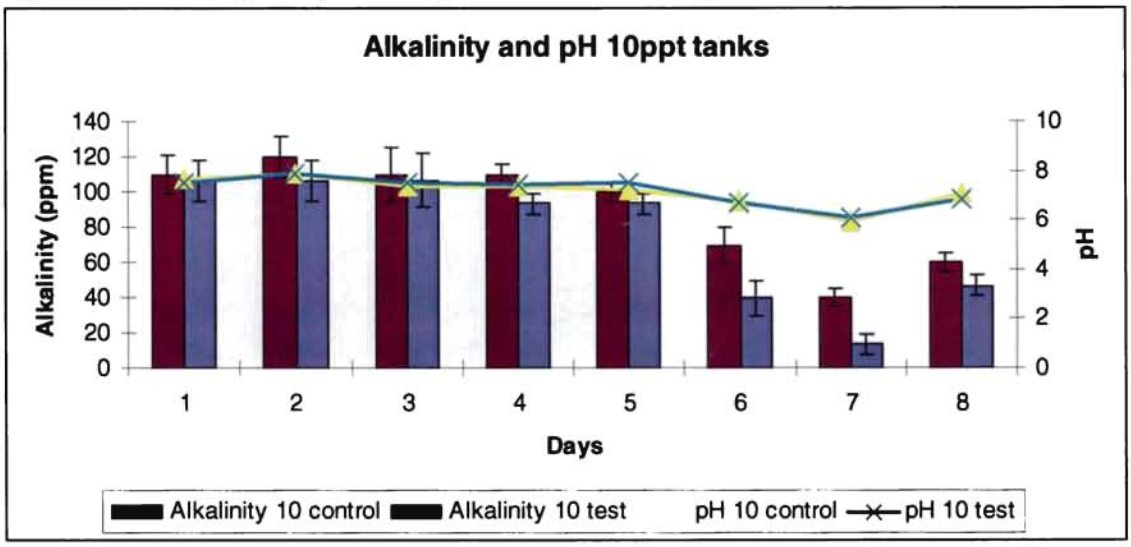
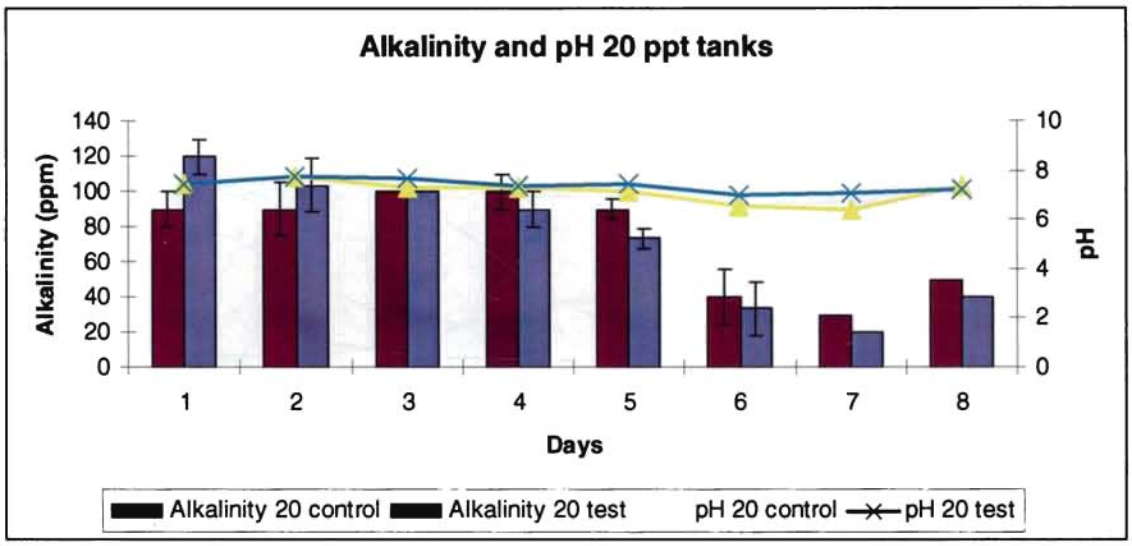


Fig. 3.15: Alkalinity and pH during TAN removal in experimental tanks.





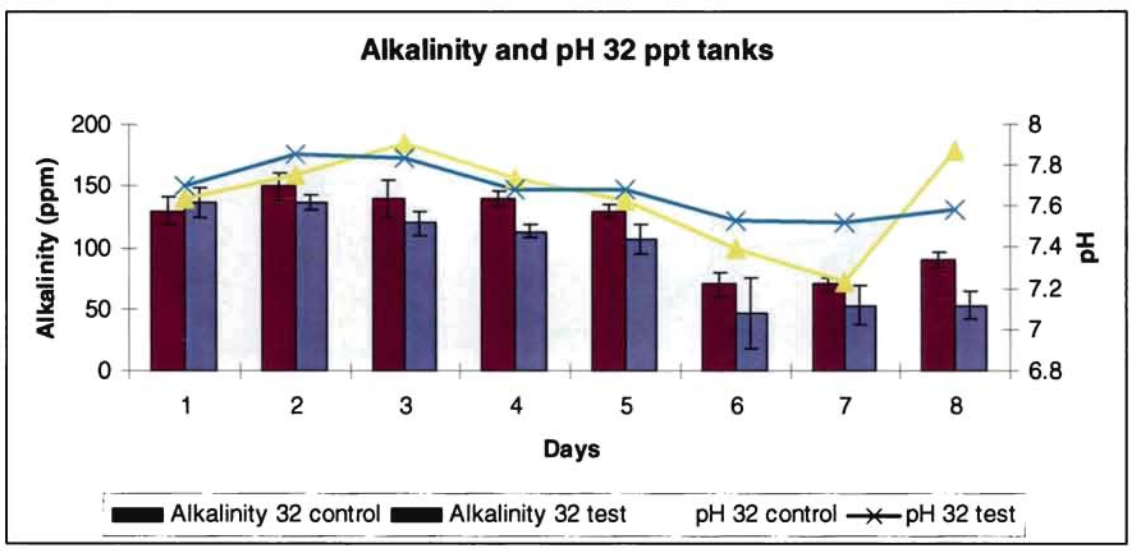
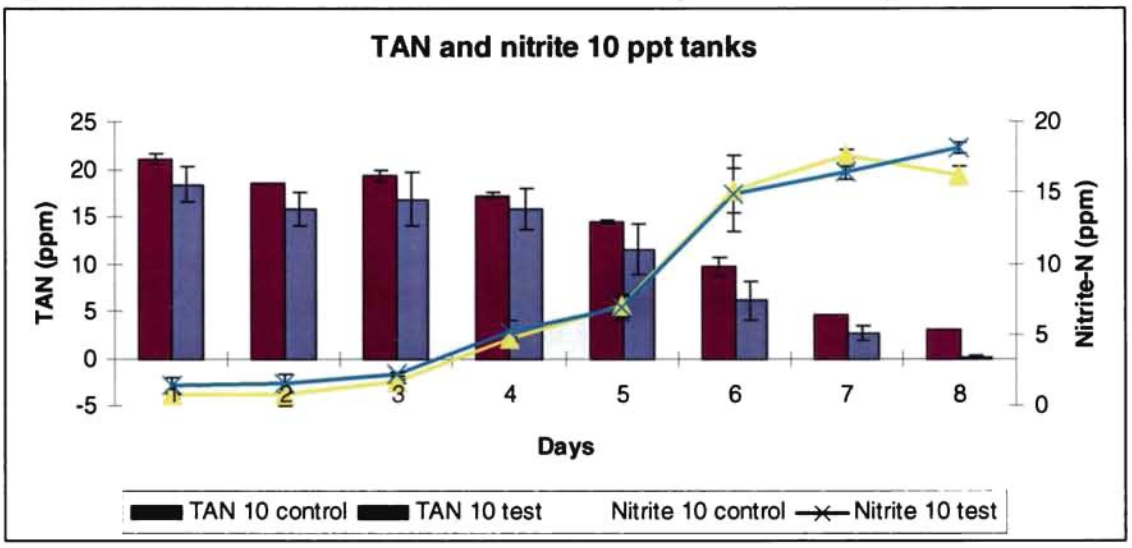
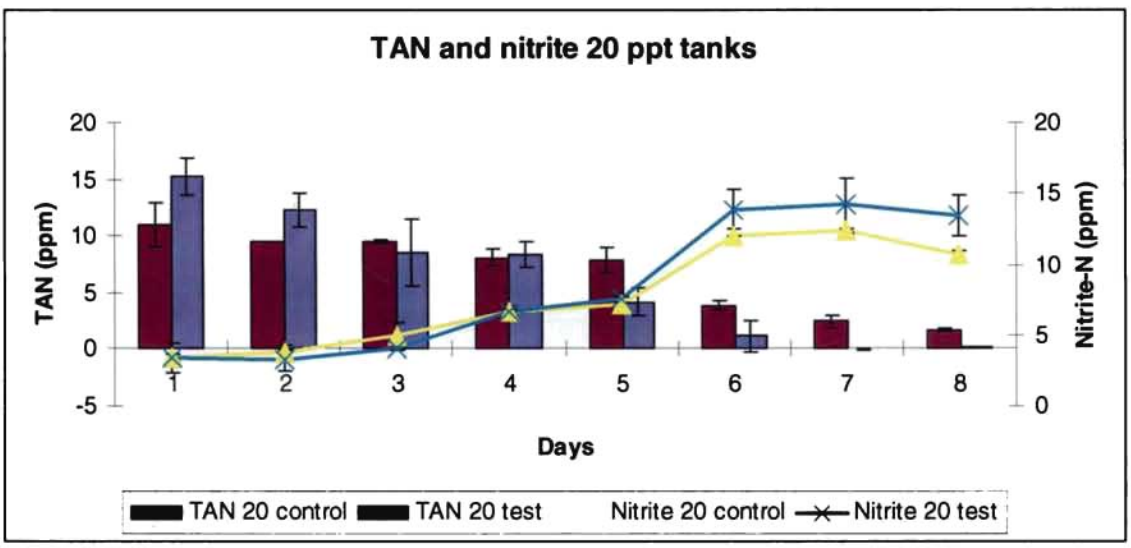


Fig. 3.16: Relation between TAN removal and nitrite production in experimental tanks.





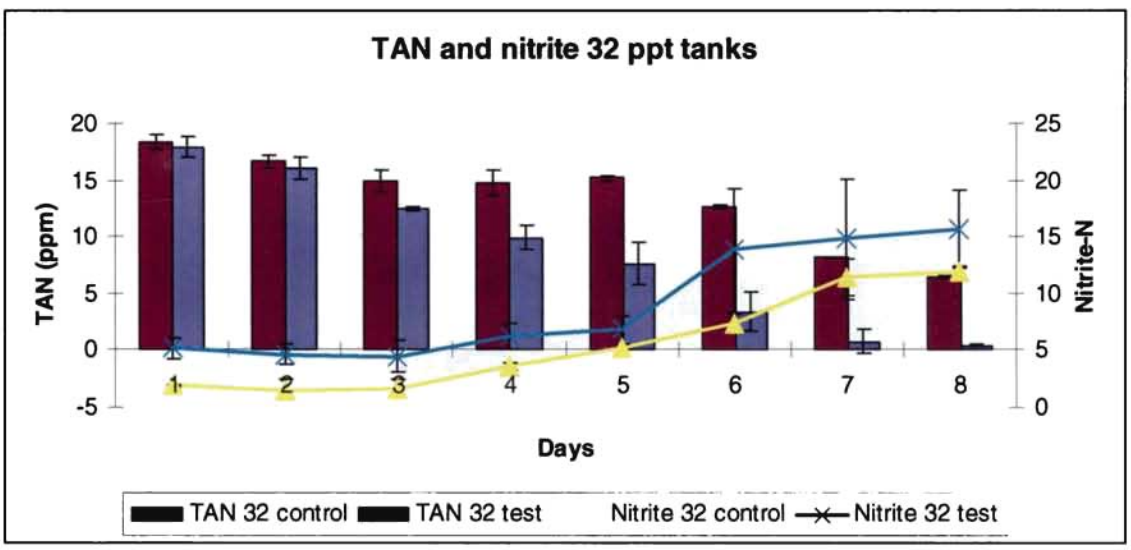
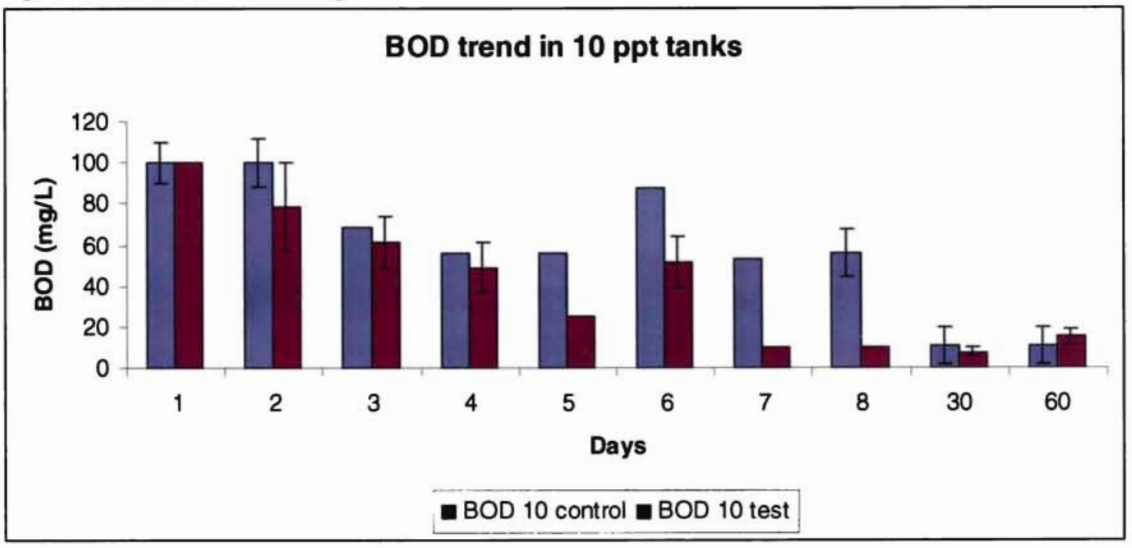
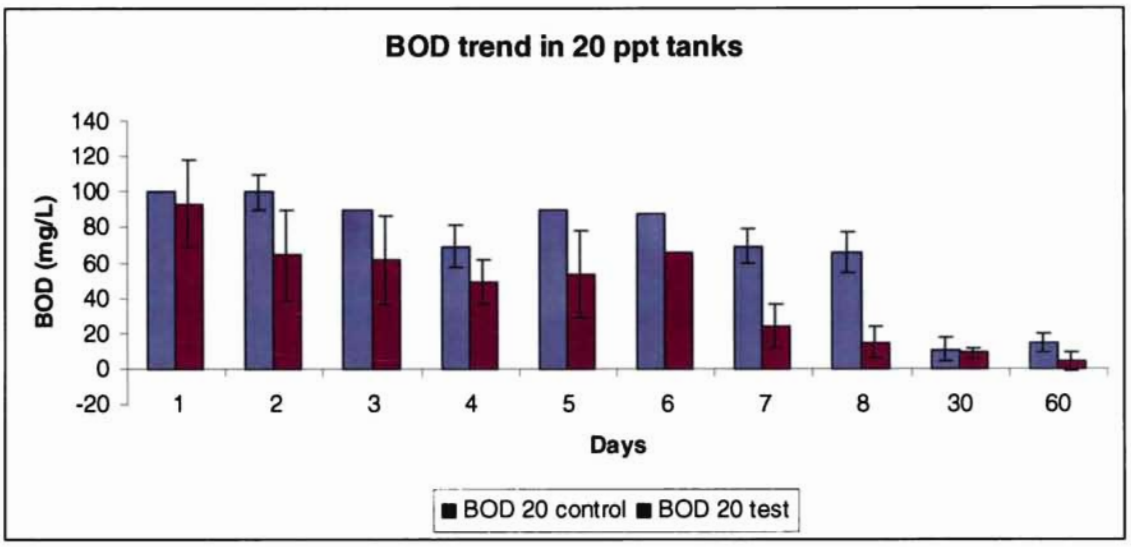


Fig. 3.17: BOD trend in experimental tanks.





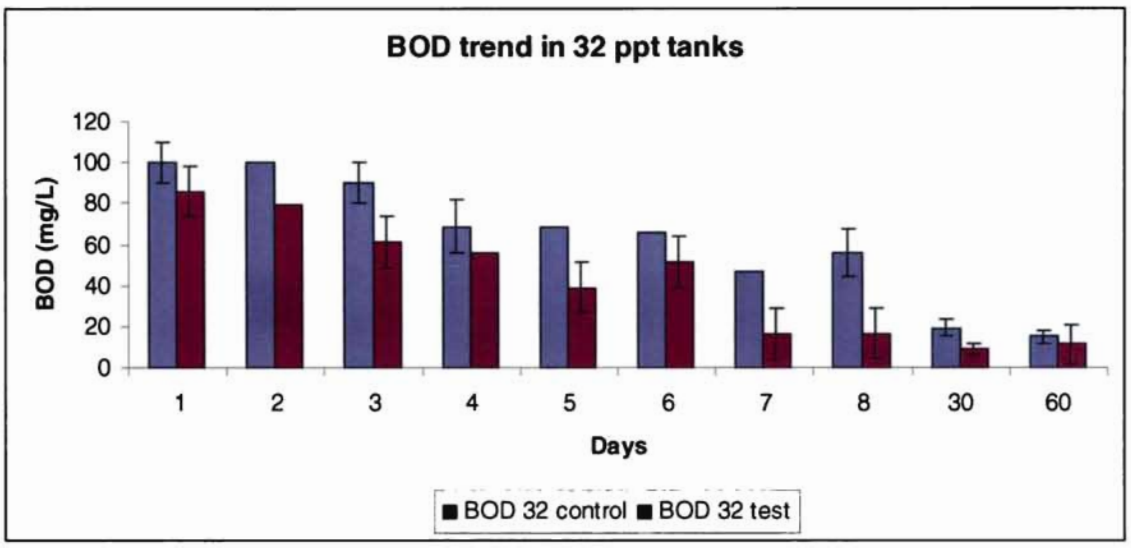


Fig. 3.18: Appearance of retrieved NBC immobilized wood powder

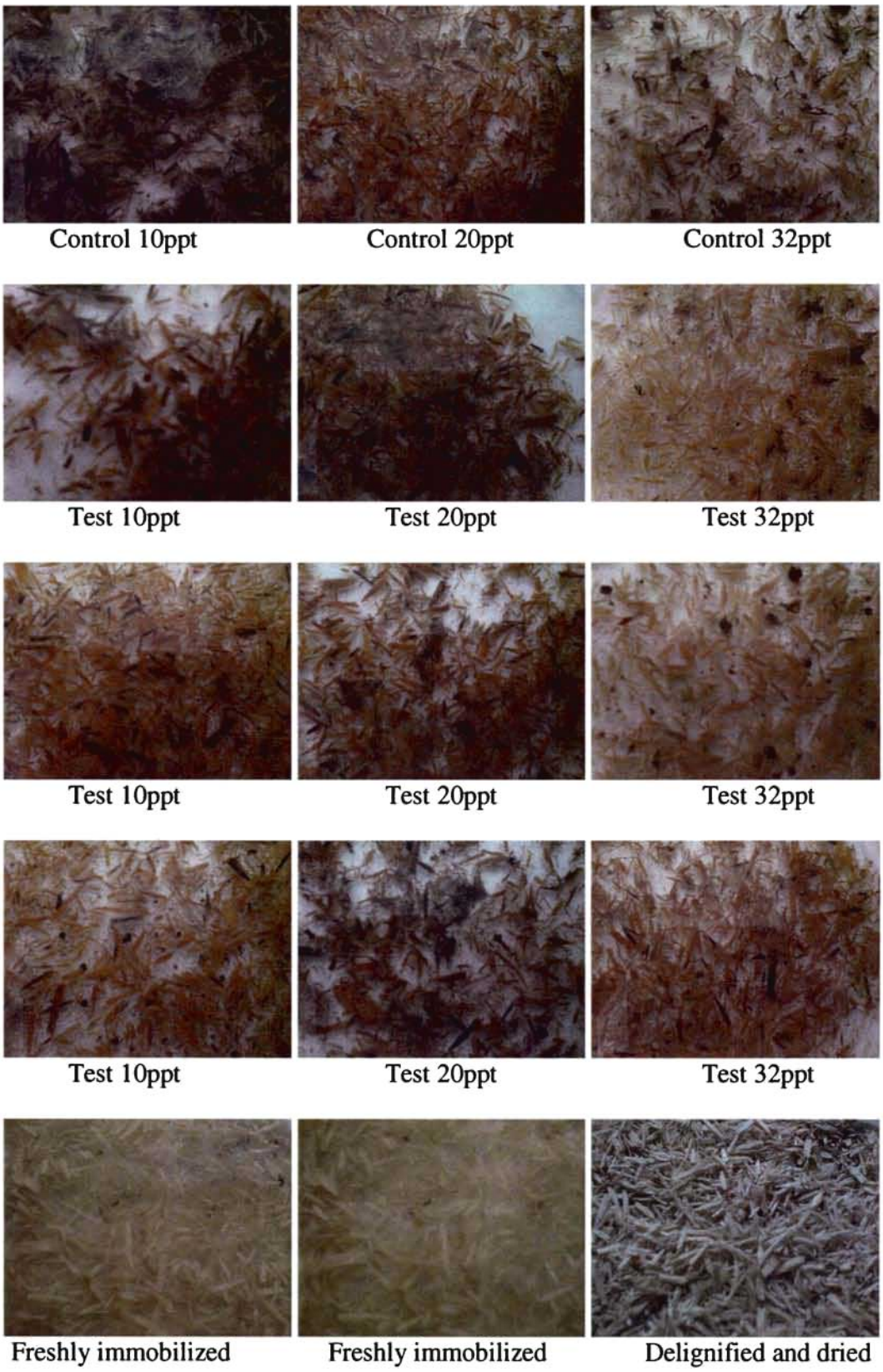


Table 3.9: Nitrifying activity during mass immobilization of AMOPCU-1.

Day of immobilization	TAN removal		NO_2-N/NO_3-N production
	(mg/L/day)	(g/day)	
Day 1	6	0.12	0.00
Day 2	4	0.08	0.00
Day 3	2	0.04	0.00
Day 4	3	0.06	0.00
Day 5	4	0.08	0.00
Day 6	2	0.04	0.00

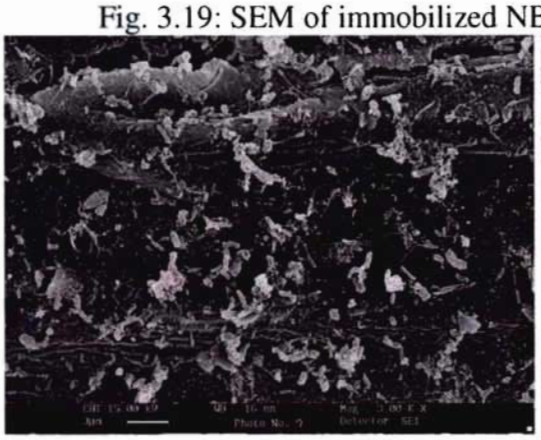
Nitrifying potency of mass immobilized consortia in fresh medium.

Sample Number		TAN Removal (mg/L/day)	TAN Removal rate (mg/day/m^2)
Day 4	Sample No. 1	4.10	0.22
	Sample No. 2	5.60	0.30
	Sample No. 3	7.30	0.39
Day 5	Sample No. 1	7.00	0.37
	Sample No. 2	5.50	0.29
	Sample No. 3	3.20	0.17

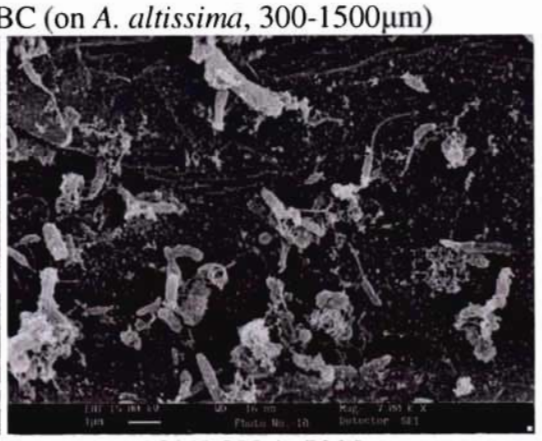
Table 3.10: Water quality of shrimp pond administered with nitrifying consortia immobilized wood powder.

Water quality parameters	Control pond Pond area (Ha): 0.4 Stocking density: 8.5/m^2		Test pond Pond area (Ha): 1.0 Stocking density: 8.5/m^2	
	Day 1	Day 2	Before addition Day 1	After addition Day 2
pH	7.40	7.89	7.40	7.67
Alkalinity (mg/L)	50	50	50	50
TAN (mg/L)	0.190	0.300	1.090	0.500
Nitrite (mg/L)	0.010	0.027	0.026	0.043
Nitrate (mg/L)	BDL	BDL	0.092	0.075

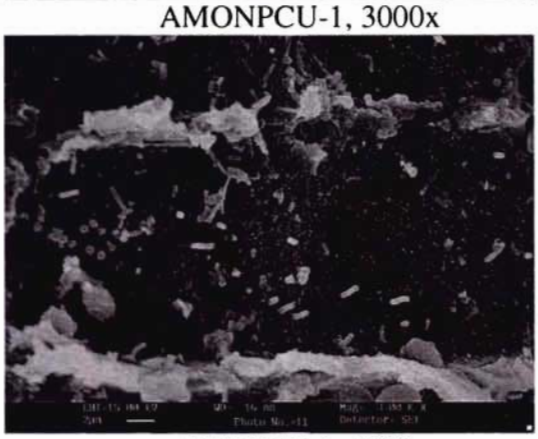
Fig. 3.19: SEM of immobilized NBC (on *A. altissima*, 300-1500μm)



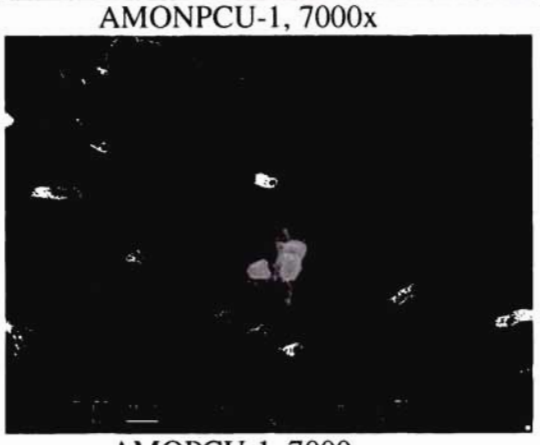
AMONPCU-1, 3000x



AMONPCU-1, 7000x



AMOPCU-1, 3000x



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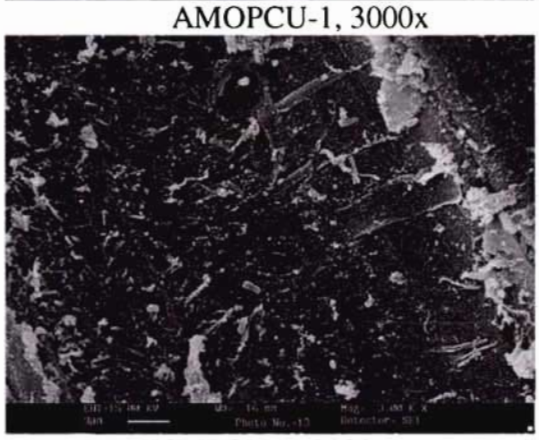

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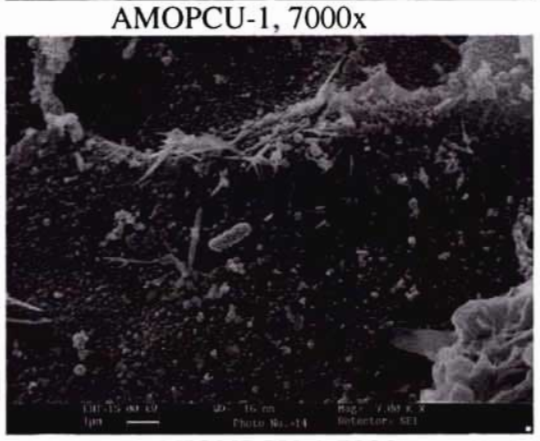
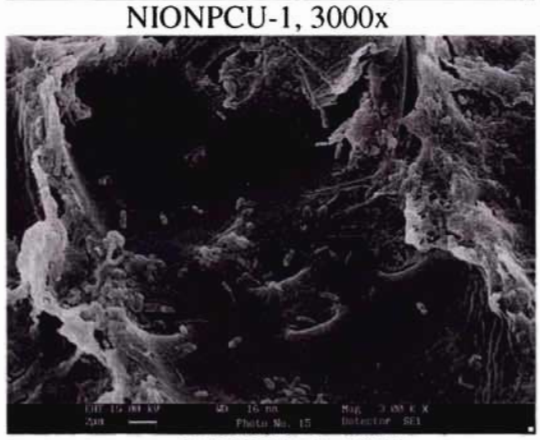
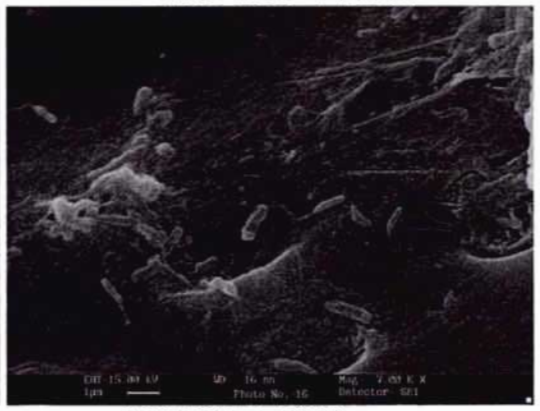


NIONPCU-1 7000x



NIOPCU-1, 3000x



NIOPCU-1, 7000x

Chapter 4

Screening, selection and partial characterization of photosynthetic sulphur bacterial consortia for bio-oxidation of hydrogen sulphide.

4.1. Introduction.

Hydrogen sulphide is one of the most toxic pollutants ranked as one of the most important inhibitor of aerobic respiration (Buisman *et al.* 1990; Kiemer *et al.* 1995). H_2S release to environment may cause plethora of problems due to its corrosive property, toxicity, unpleasant odour and heavy oxygen demand. Hydrogen sulphide, the most reduced form of sulphur, has a high oxygen demand of 2mol O_2 mol^{-1} H_2S (Kobayashi *et al.* 1983). Conventional methods commonly adopted for the removal of sulphide are based on physico-chemical processes. Direct air stripping, oxidation and chemical precipitation are the commonly used strategies, but are expensive in terms of energy, chemicals and disposal of spent chemicals (Buisman *et al.* 1990). Oxidation requires use of oxidizing agents like air, oxygen chlorine, potassium ferricyanide, quinone, manganese dioxide, manganese sulphide, hypochlorite, chlorine dioxide and nitric oxide (Kobayashi *et al.* 1983). These methods require physical treatments like aeration, heating and centrifugation. Use of chemical additives generates chemical waste that will contaminate environment if disposed with out proper treatment.

H_2S removal by microbiological methods (photosynthetic as well as non-photosynthetic microorganisms) is a good alternative to physico-chemical treatments because of its economic feasibility. Biotechnological sulphide removal system is based on the oxidation of sulphide to sulphur. Photosynthetic purple and green sulphur bacteria utilize reduced sulphur compounds, such as sulphide, as electron donors (Dahl *et al.* 1999). H_2S can be converted to elementary sulfur or sulfate by the photosynthetic bacterium *Chlorobium thiosulfatophilum*, which would be a feasible option for biological sulfide removal because of its relatively high rate, and simple nutrient requirements (Lee and Kim, 1998). Elementary sulfur is formed as an intermediate, or sulfate may be the end product of such processes (Dahl *et al.* 1999) and such methods are more viable because of its high conversion rate, less demand on nutrients, non-requirement of oxygen and sterilization (Lee and Kim, 1998). As per Buisman *et al.* (1990) biotechnological sulphide removal has several merits,

a) Catalyst and oxidants are not used, making the process economical.
b) No hassles of chemical sludge disposal.
c) Biological sludge production is at bare minimal level.
d) Low energy consumption.
e) Possible reuse of sulphur, which is a byproduct of the process.
f) Low sulphate and thiosulphate discharge.

g) Fairly fast and high removal efficiency.

4.1.1. Hydrogen sulphide production in aquatic systems.

Accumulated organic matter in an aquaculture pond leads to oxygen depletion in the vicinity of sediment-water interface. With the depletion of oxygen, decomposition of organic matter would proceed in the presence of other electron acceptors other than oxygen. High rate of mineralization often leads to oxygen depletion in sediments and much of the mineralization therefore takes place under anoxic conditions. Carbon oxidation under anoxic conditions occurs with NO_3^-, MnO_2, FeO(OH), SO_4^{2-}, and CO_2, which leads to a vertical stratification of microbial respiration in the sediments (Holmer and Storkholm, 2001). This shift triggers off anaerobic processes in the pond bottom, which leads to the production of reduced and potentially toxic compounds (Avnimelech and Ritvo, 2003). Anaerobic degradation of organic matter is a complex process mediated by a consortium of physiologically different microorganisms and is less energetically favourable than aerobic decomposition. Sulphide produced during sulphate reduction may undergo reoxidation by several processes like chemical oxidation with oxygen, anoxic chemical oxidation, phototrophic oxidation, bacterial oxidation under oxic and anoxic conditions (Holmer and Storkholm, 2001). Hydrogen sulphide can be produced at micromolar to millimolar levels under normal conditions, in shallow waters, salt marshes, enclosed bays and estuaries. Most of the aquatic fauna avoid such habitats, though a very few are sulphide tolerant and most pelagic species are sulphide-intolerant (Kiemer *et al.* 1995). Accumulation of dissolved sulphide may reduce recolonization of benthic fauna as a result of toxicity and this may affect the restoration of lakes/ water bodies after eutrophication events (Holmer and Storkholm, 2001).

4.1.2. Hydrogen sulphide in aquaculture.

In aquaculture reservoirs, sulphide production occurs in anoxic sediments due enhanced sulphate reduction. Due to the inherent high concentration of sulphate in sea water used in shrimp culture, potential sulphide production will be high due to the action of sulfate reducing bacteria. Sulphide oxidation contributes majority of sediment oxidation demand in shrimp ponds; at the end of culture cycle it was reported to be 84% of the total sediment oxidation demand (Suplee and Cotner, 1996). Accumulated sludge in a shrimp pond has a characteristic black colour with hydrogen sulphide smell. Even small concentrations of sulphides must be avoided in commercial aquaculture (Lahav, *et al.*

2004). Hydrogen sulphide affects egg and fry development of many commercially cultured fishes (Boyd, 1995). In salmon cage culture, a phenomenon termed 'site souring' has been reported, which describes a site where fish performance is reduced over time with slower growth and increased susceptibility to diseases (Kiemer *et al.* 1995). Such phenomenon was attributed to the organic enrichment of sediments especially sulphide rich out-gassing sediments beneath fish cages. In the year 2000 mass fish mortality (400 Ton) occurred in 40 ha Einan reservoir, Israel, due to sulphide toxicity, subsequent oxygen depletion and destratification (Lahav *et al.* 2004). Sulphide also inhibits nitrification, which would obviously leads to ammonia toxicity in aquaculture ponds (Avnimelech and Ritvo, 2003). Oxidation of hydrogen sulphide is very important in aquaculture ponds as black precipitation due to hydrogen sulphide results in gill clogging which would adversely affect shrimp health and growth (Devaraja *et al.* 2002).

4.1.3. Hydrogen sulphide toxicity.

Unionized hydrogen sulphide is extremely toxic to aquatic organisms even at very low concentrations, but its dissociation products are not appreciably toxic. The proportion of unionized hydrogen sulphide decreases with increase in pH (Boyd, 1995). The threshold limit value for fresh or salt water fish is 0.5ppm (Henshaw and Zhu, 2001). Undissociated hydrogen sulphide inhibits aerobic respiration by binding to the heme of cytochrome *c* oxidase instead of molecular oxygen. HS^- has a strong affinity for the ferric ion bound to the heme of the cytochrome, the enzyme cannot be reduced back to the ferrous oxidation state and this prevents oxygen release by oxyhemoglobin (Kiemer *et al.* 1995). Resulting cellular anoxia prevents ATP production and divert metabolism to lactic acid production. Hydrogen sulphide toxicity is inversely related to dissolved oxygen concentration, and presence of sulphide aggravates sensitivity to hypoxia. Affonso *et al.* (2004) reported that cytochrome *c* oxidase activity was very less in the gills and blood of air-breathing catfish (*Hoplosternum littorale*) in the presence of high levels of dissolved sulphide. Atlantic salmon smolts (*Salmo salar* L.) when exposed to both chronic and acute sub lethal levels of hydrogen sulphide developed liver damage (Kiemer *et al.* 1995). Gopakumar and Kuttyamma (1997) reported that *Litopenaeus indicus* avoids pond bottom regions with sulphide containing sediment and feed acceptance was considerably low in such sediments. The 48h LC_{50} for H_2S in *Metapenaeus monoceros* zoea, mysis and juvenile stages were 0.008, 0.012 and 0.019mg l^{-1} repectively. When H_2S concentration in the hemolymph reached 0.013mg l^{-1} shrimp became paralysed (Avnimelech and Ritvo,

2003). The 96h LC_{50} of H_2S was higher at lower pH and toxic concentration ranged from 0.063 to 0.342mg l^{-1} for *Penaeus indicus* and from 0.077 to 0.378mg l^{-1} for *M. dobsoni* (Gopakumar and Kuttyamma, 1996).

4.1.4. Mitigation of hydrogen sulphide in aquaculture: Present scenario.

Hydrogen sulphide menace has been sidelined or overlooked largely due to the efforts for ammonia detoxification in aquaculture as it is the second most important factor affecting intensive aquaculture production. In well aerated intensive aquaculture systems hydrogen sulphide may not be a problem, but in Indian context where semi intensive version is more prevalent, it assumes importance as a critical factor affecting production both in quantity and quality. Options for hydrogen sulphide amelioration in aquaculture are rather narrow, comprehensive technology to mitigate sulphide problem has not been addressed yet and very few studies were conducted to minimize the detrimental effects of hydrogen sulphide. Practical approaches for controlling sulphide concentration in aquaculture systems can be (i) Increasing the redox potential in the pond by chemical (oxygen or nitrate) poising, which minimize the effect of sulphate reducing bacteria and thus sulphide formation. This is possible only if there is high oxygen and nitrate concentration in the bulk of water above the sediment. (ii) Sulphide removal by chemical oxidation, precipitation of insoluble metal sulphide or a combination of both (Lahav *et al.* 2004). But these options are not cost effective due to high chemical cost and most of the chemical oxidizing agents cannot be used in aquaculture. Potential use of soil rich in iron for controlling hydrogen sulphide concentration in freshwater aquaculture systems was studied by Lahav *et al.* (2004). In this study, it was hypothesized that addition of iron rich soil prior to seasonal de-stratification of fish farming reservoirs would significantly reduce the toxic effects and sediment oxygen demand associated with sulphide.

4.1.5. Biological sulphide removal.

Anoxygenic photosynthesis is one of the major biological processes of the sulphur cycle. In benthic systems, the sulphide production resulting from the activity of sulphate reducing bacteria can be very intense, especially in microbial mats. The biological oxidation of sulphide to sulphate is performed by two main groups of bacteria, the sulphur oxidizing bacteria and the phototrophic sulphur bacteria. The chemical oxidation of sulphide is a rather slow process that can be enhanced in sediments by the presence of trace metals, e.g. iron and manganese. If sufficient light is present and oxygen levels are

low, anoxygenic photosynthesis is the main microbiological pathway of sulphide oxidation (Pringault *et al.* 1999). Blooms of phototrophic sulphur bacteria have been reported from a wide variety of anoxic environments, where reduced sulphur compounds are plenty along with light. Stratified lakes, coastal lagoons, estuaries, eutrophic shallow forest ponds, sediments of rice fields and coastal mangrove swamps are common habitats of these microorganisms. They can also be found in extreme environments like salt lakes, soda lakes and hot sulphur springs (Casamayor *et al.* 2000). In many microbial mats dominated by oxygenic phototrophs, it is more likely to see underlying or intermixed layers of anoxic phototrophic organisms. Steep difference in redox and chemical gradients that establish in phototrophic biofilms and mats enforce such stratification (Roeselers *et al.* 2007).

Although numerous forms of photosynthetic bacteria exist, only members of the groups of *Chromatiaceae* (Purple sulfur Bacteria) and *Chlorobiaceae* (green sulfur bacteria) are commonly known to metabolize sulfide. Sulfide metabolism by photosynthetic sulfur bacteria occurs only under anaerobic conditions, and is coupled to CO_2 reduction. Sulfide serves as the sole electron donor and is oxidized to sulfate either directly, or by way of elemental sulfur, as is the case with *Chlorobium* and *Chromatium,* members of *Chlorobiaceae* and *Chromatiaceae* respectively (Kobayashi *et al.* 1983).

Biological oxidation of sulphide to sulphate is a two-step reaction. In the first stage, which proceeds faster than the second stage, sulphide looses two electrons and membrane bound polymeric sulphur compounds are formed. Photosynthetic reaction center accept electrons by oxidation of H_2S to elementary sulphur. In the second step, sulphide oxidation is via sulphite to sulphate only in the case of electron deficiency.

$$2H_2S + CO_2 \xrightarrow{\text{light}} 2S^0 + (CH_2O) + H_2O \qquad (1)$$

$$H_2S + 2CO_2 + 2H_2O \xrightarrow{\text{light}} 2(CH_2O) + H_2SO_4 \qquad (2)$$

Stronger light intensities and lower sulphide feed rate, result in further oxidation of sulphur to sulphate; due to demand for more electrons from any available species of sulphur with oxidation state less than +6 (Lee and Kim, 1998). Thus sulphate is formed only when the concentration of sulphide is less.

$$(H_2S)\ S^{-2} \text{ ——first step——} \rightarrow S^0 \text{——second step——} \rightarrow S^{+6}\ (H_2SO_4)$$

Aerobic sulphide oxidation occurs by colorless sulphur bacteria but they often use thiosulphate rather than sulphide and in this case sulphate is generally formed as the end product. The following overall reactions occur in an aerobic sulphide removal system:

$$2HS^- + O_2 \longrightarrow 2\ S^0 + 2OH^- \qquad (3)$$

$$2\ S^0 + 3O_2 \longrightarrow 2SO_4^{2-} + 2H^+ \qquad (4)$$

4.1.6. Anoxygenic phototrophs for sulphide removal.

Chemotrophic sulphide-oxidizing microorganisms using either oxygen or nitrate as ultimate electron acceptor can be used for sulphide removal. Aerobic sulfur-oxidizing bacteria like *Thiobacillus* is used in aerobic bioreactors and such systems cannot be integrated with anaerobic treatments (Roeselers *et al.* 2007). Hence anaerobic oxidation by phototrophic sulphur bacteria like *Chloroflexi*, green and purple sulphur bacteria holds promise in this regard and it has been proposed as an alternative method for sulphide removal (Kim *et al.* 1990). Purple and green sulphur bacteria are phylogenetically coherent groups, distantly related each other.

4.1.7. Phototrophic sulphur bacteria.

Phototrophic sulphur bacteria are commonly found in illuminated aquatic environments containing hydrogen sulphide. They thrive in the illuminated parts of anoxic water (Pfennig and Truper, 1981), and in the top few millimeters of shallow aquatic sediments (Pringault *et al.* 1998). One of the reasons for anaerobic preference may be related to their photosynthetic reaction centers which produce low-potential reductants including reduced ferredoxin (Frigaard and Matsuura, 1999). The growth of phototrophic sulphur bacteria in benthic systems is restricted to well-defined layers within the sedimentary oxygen, sulphide, pH and light gradients; in these gradients where parameters vary within millimeters, phototrophic microbial mats can reach several millimeters, in thickness (Pringault *et al.* 1996). It is assumed that a larger diversity of phototrophic sulphur bacterial community exist in the chemocline of lakes as most of the enrichments of green and purple sulphur bacteria did not hybridize with the established probes (Tonolla *et al.* 2005).

4.1.7.1. Green sulphur bacteria.

Green sulphur bacteria are obligate photoautotrophs, found in anoxic and sulphide rich waters, muds, sediments and microbial mats. Growth is by photooxidation of reduced sulphur compounds in the absence of oxygen, but they may encounter oxic conditions in their natural habitats (Frigaard and Matsuura, 1999) due to disturbance in vertical stratification of water bodies and sediments. However, they are extremely sensitive to molecular oxygen and growth is entirely inhibited by trace amounts of molecular oxygen (Pringault *et al.* 1998). Green sulphur bacteria have a very high affinity for sulphide; they tolerate and grow at high sulphide concentrations (5-10mM), which are toxic to most purple sulphur bacteria. Green sulphur bacteria only need low light flux for growth and maintenance (Pringault *et al.* 1998). Green sulphur bacteria use an efficient light harvesting structure called chlorosomes, attached to the cytoplasmic side of cellular membrane, and aerobic conditions inhibits energy transfer from such light harvesting system (Frigaard and Matsuura, 1999). Bacterial chlorophyll molecules are located inside the chlorosomes and bchl *c, d* or *e* are the main light harvesting pigments in anoxic green sulphur bacteria (Borrego *et al.* 1998). Bacteriochlorophyll distribution in the Black Sea was studied by Repeta *et al.* (1989) and found that anoxic photosynthesis by brown sulphur bacteria *Chlorobium phaeovibrioides* and *Chlorobium phaeobacterioides* was prevalent in world's largest anoxic marine water, The Black Sea.

4.1.7.2. Purple sulphur bacteria.

They are commonly encountered in anoxic water containing reduced sulphur compounds and light. Several species belonging to *Chromatiaceae* have been isolated from different environments. These strains mainly belong to *Chromatium* species (*C. vinosum, C. gracile, C. purpuratum)* and to *Thiocapsa roseopersicina,* species commonly isolated from eutrophic lagoons (Guyoneaud *et al.* 1997). *Thiocystis* comprises coccus shaped organisms devoid of gas vesicles and motile representatives of the family *Chromatiaceae*; whereas *Thiocapsa* are non motile. Purple sulphur bacteria requires more light and have lower sulphide tolerance than green sulphur bacteria; and are usually found above green sulphur bacteria (Casamayor *et al.* 2001). These bacteria are found abundantly in sulphur springs and mud water.

4.1.8. High rate photosynthetic systems in aquaculture.

Recently much emphasis is given for partitioned aquaculture system (PAS) combining various physical, chemical and microbial intensification techniques in a single integrated system. Photosynthetic suspended growth systems couple natural processes like photosynthesis, algal nutrient uptake, nitrification-denitrification, and organic matter oxidation; with mechanical aids for aeration and water circulation (Hargreaves, 2006). High rate micro algal culture to produce a sustainable, high yielding, controlled production with minimal discharge is the central theme of such concept (Brune *et al.* 2003). However, such a concept was mooted for pushing the ceiling of ammonia detoxification rate in a system in particular, or to enhance nitrogen cycling in intensive aquaculture systems. Use and manipulation of photosynthetic microbial community for the hydrogen sulphide amelioration is still a nascent area in intensive aquaculture.

4.1.9. Application of photosynthetic green and purple sulphur bacteria in zero exchange shrimp grow outs.

In zero water exchange shrimp grow outs, hydrogen sulphide toxicity is one of the major issues to be addressed through bioremediation. It has been conceptualized to address this problem through bioaugmentation employing photosynthetic sulphur bacteria. This chapter deals with enrichment, screening, partial characterization and designing of bench top photobioreactor for determination of its kinetics.

4.2. Materials and methods

4.2.1. Enrichment of Photosynthetic sulphur bacteria.

Water samples were collected from different localities; samples include pond water and sediment collected from Ponneri in Tamil Nadu, Kodungallore, Maradu, Cochin areas in Kerala, Nelloor in Andra Pradesh, Goa and mangrove sediment samples from Minicoy Island, Union territory of Lakshadweep. Salinity in the sampling stations varied between 10 - 40.

Samples were enriched in Pfennig's Medium I (Modified 1988, for Purple sulphur bacteria) and Pfennig's Medium II (modified 1988, for green sulphur bacteria) prepared in different salinity regimes (5, 15, 25 and 40) in order to acclimatize it in varied environmental conditions. As alkalinity and hardness are very much correlated with

salinity, the suitability of an organism in different alkalinity and hardness regimes could also be tested by this process.

Pfennig's Medium I (Modified 1988, for Purple sulphur bacteria).

Solution A (should be prepared fresh)	
Ingredients	
$CaCl_2$. 2 H_2O	1.25g
KH_2PO_4	1.7g
NH_4Cl	1.7g
KCl	1.7g
$MgSO_4$	2.5g
Distilled water	4860.0mL

(By adding all ingredients the volume will be 4990mL. Supplementary solutions B, C, D & E can be prepared and maintained.

Solution B	
Vitamin B_{12} : 0.002% in H_2O (Filter sterilized solution maintained at 4^0C)	5.00mL

Solution C		
Trace element solution		5.00mL
Ingredients.		
Na_2-EDTA	3.00g	
$FeSO_4$ X $7H_2O$	1.10g	
$CoCl_2$ X $6H_2O$	190.00mg	
$MnCl_2$ X $2H_2O$	50.00mg	
$ZnCl_2$	42.00mg	
$NiCl_2$ X $6H_2O$	24.00mg	
Na_2MoO_4 X $2H_2O$	18.00mg	
H_3BO_3	300.00mg	
$CuCl_2$ X 2 H_2O	2.00mg	
Distilled water	1000.00mL	
Adjusted pH to 6.0 & Autoclaved at 121°C for 15 min. Maintained at 4°C.		

Solution D		
$NaHCO_3$	7.50g	100.00mL
H_2O	100.00mL	
Filter sterilized and saturated with filtered CO_2. Store at 4°C.		

Solution E	
Na_2S (3.25g in 100mL) Autoclaved and stored at 4°C.	20.00mL

Neutralized sulphide solution was prepared by dissolving 0.5g Na_2S in 100mL distilled water bubbled with nitrogen gas and autoclaved for 15 min. at 121°C. After cooling to room temperature, the pH was adjusted to about 7.3 by adding sterile 2M H_2SO_4 drop wise

with a syringe without opening the bottle. Appearance of a yellow colour indicated the drop of pH to about 8.0. The solution was stirred continuously to avoid precipitation of elemental sulphur. The final solution was clear and yellow in colour. Subsequently, the medium was stored at 4°C.

After autoclaving, solution A was cooled to room temperature, and saturated with CO_2 by magnetic stirring for 30 minutes under a CO_2 atmosphere of 0.05-0.1atm. Solutions B, C, D & E dispensed with a Pasteur pipette fitted with a cotton plug against a stream of N_2 gas while the medium was stirred with a magnetic stirring bar. Finally pH adjusted with sterile HCl or Na_2CO_3 solution (2mol/litre each) to pH 7.3.

1g each (spatula full) of soil samples were introduced into screw capped tubes and the media were poured to fill up the volume. The tubes were closed tightly and incubated in light. Periodically, cultures were supplemented with neutralized solution of sodium sulphide (0.005 to 0.01 % end concentration) to replenish sulphide.

Pfennig's Medium II (modified 1988, for green sulphur bacteria)

Following alterations were made to Pfennig's medium I to prepare Pfennig's medium II. Trace element solution composition was different in Pfennig's medium II.

Solution C (trace element solution for Pfennig's medium II)		
Trace element solution		5.00mL
Ingredients.		
HCl (25%)	7.7mL	
$FeSO_4$ X $7H_2O$	1.50g	
$CoCl_2$ X $6H_2O$	190.00mg	
$MnCl_2$ X $2H_2O$	100.00mg	
$ZnCl_2$	70.00mg	
$NiCl_2$ X $6H_2O$	24.00mg	
Na_2MoO_4 X $2H_2O$	36.00mg	
H_3BO_3	300.00mg	
$CuCl_2$ X 2 H_2O	2.00mg	
Distilled water	1000.00mL	
Adjusted pH to 6.0 & Autoclaved at 121°C for 15 min. Maintained at 4°C.		

Solution E	
Na_2S (3.25g in 100mL) Autoclaved and stored at 4°C.	30.00mL

After combining and mixing all solutions (as in the case of medium I) pH adjusted to 6.8, with sterile HCl or Na_2CO_3 solution (2mol/litre each).

4.2.2. Screening of photosynthetic sulphur bacterial consortia for their potency to remove sulfide

136 isolates of photosynthetic sulphur bacterial consortia enriched from different environmental conditions were screened for their potency to remove hydrogen sulphide. Fresh sulphide stock solution was prepared and added to the culture maintained in sealed screw cap bottles to the final concentration of 1ppm. After 24 hour, sulphide was measured by methylene blue spectrophotometric method as described by Fonselius (1983). Difference in sulphide concentration during the period of incubation was accounted for calculating removal rate.

4.2.3. Standardization of sonication for maximum cell dispersion of photosynthetic sulphur bacterial consortia.

Quantitative determination of the bacteria in the consortia which form larger aggregates requires reliable methods for efficient, nondestructive de-aggregation of biofilm and flocs. Ultrasonication was reported to be a suitable technique for desegregation of bacterial flocs. Treatment periods and power varied during various ultrasonic treatments to determine the condition at which greater number of single cells obtained as enumcrated by epifluorescence microscopy.

4.2.4. Enumeration of photosynthetic sulphur bacterial consortia by epifluorescence microscopy.

Sonicated bacterial samples were stained with acridine orange and filtered onto a 0.2μm irgalan black stained nuclepore polycarbonate filter (Millipore GTBP011300). To get uniform cell distribution, wetted the base of the filtration apparatus prior to placement of the wet polycarbonate membrane. After filtration, nuclepore filters were mounted immediately on a slide using non-fluorescent immersion oil. A minimum of seven fields per filter per sample were counted. Bacterial cells fluoresce green taking up acridine orange under blue excitation on an epifluorescence microscope. Fluorescing images less than 0.2μm in diameter can be disregarded. An eyepiece of known area was used during enumeration.

Calculation:

$$\text{Bacterial abundance (cells/liter)} = C_f R \times 1/Fs$$

C_f = mean number of cells /field.

R = active area of filter/ area of field counted.

Fs = volume of water filtered (litre).

4.2.5. Amplification of the segregated photosynthetic sulphur bacteria in photobioreactor.

For amplification of the selected photosynthetic sulphur bacterial consortia a 5L glass fermentor (B-Lite, Sartorius. India) was modified to a photobioreactor. An illuminating system was used with a metal halide lamp (Osram, Concentra PAR38 Flood, 80W, 240V, Osram, Netherland). Light intensity on the surface of the reactor was measured with a light intensity meter (TES-1330, digital illuminance meter, TES electrical electronic corp., Taiwan) and maintained at 1000 lux. The reactor was filled with 3L Pfennig medium II (for green sulphur bacteria) and anaerobic conditions were maintained in the reactor. The liquid medium had previously been prepared under nitrogen atmosphere. Water was circulated into the external jacket of the reactor for controlling the temperature. Temperature was maintained at 27°C and 29°C, pH maintained at 6.8 and 7.3 for green and purple sulphur bacteria respectively. The contents of the reactor were completely mixed at 200 rpm. Zinc acetate traps were maintained in the exhaust line to scrub any H_2S gas which was not absorbed in the medium. The schematic diagram of the reactor system is shown in Fig. 4.1 (photographs in Fig. 4.2). Green and purple sulphur bacterial consortia were inoculated in photobioreactor. Initial cell loadings were 10^9 cells/mL. Sulfide concentration was maintained at 1ppm during the entire culture period. Biomass generated in the photobioreactor was quantified and sulfide removal rate per unit biomass was calculated. Sulphide was measured by methylene blue spectrophotometric method as described by Fonselius (1983).

4.2.6. Characterization of segregated photosynthetic sulphur bacterial consortia.

4.2.6.1. Transmission electron microscopy.

Cultures were centrifuged (4000g, 10min. at 4°C) and the pellets fully suspended in the primary fixative solution. During each further stage of the processing before dehydration, the cells were centrifuged (12000g, 2min. at 4°C) and the pellets were suspended carefully in the next reagent. The primary fixative used contained 75mM lysine in buffered 0.075% (w/v) ruthenium red, 2% (w/v) paraformaldehyde and 2.5% (w/v) gluteraldehyde for extended time of 24hr (Fassel *et al.* 1998). Pellets were washed three times for 10min. in 0.1M sodium cacodylate buffer (pH 7.2). Post fixation was in 1% (w/v) OsO_4, 0.1M sodium cacodylate buffer (pH 7.2) for 2hr. Resulting pellets were washed

three times for 10minutes, in 0.1M sodium cacodylate buffer (pH 7.2) and dehydrated. The dehydration was done 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 electron microscope (Morgagni 268-D, Netherlands).

4.2.6.2. Absorption spectra measurement

5g of sucrose was mixed with 3.5mL of cell suspension until the dissolution of sucrose was complete. Then the spectrum was recorded against a blank of 5g of sucrose in 3.5mL of water using a UV-Visible spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, Tokyo, Japan.) (Truper and Pfennig, 1981). The characteristic spectrum was analyzed for specific absorption peaks and compared with the documented ones.

4.2.6.3. Flourescent *in situ* hybridization

Hybridizations were performed on 6-well, Teflon-coated slides (Electron Microscopy Sciences, USA). After centrifugation, bacterial cells were fixed at 4°C by adding 2 vol. of fixative [4% paraformaldehyde in 1X PBS (130mM NaCl, and 30mM Na-phosphate, pH 7.2)] to 1 vol. of cell suspension. Cell smears were prepared by applying 10µL of the cell suspension (1.0 X 10^8 cells mL^{-1}) on each spot of the hybridization slides and dried. Samples were then dehydrated in an ethanol series (50, 80 and 100%, 3 minutes each). For hybridization, 10µL hybridization buffer [0.9M NaCl, 20mM Tris-HCl (pH 7.2), 0.01% SDS and 10-40% Formamide (depending on the probe)] and 1µL oligonucleotide probe (25ng μL^{-1}) were applied to each spot and hybridization was carried out for 2h at 46°C. Oligonucleotide probes used (Table 4.1) for the study were GSB-532 (10% Formamide required) targeting green sulphur bacteria (Tuschak *et al.* 1999), S-F-Chrom-986-b-A-20 specific for members of Chromatiaceae (Bosshard *et al.* 2000) and S453F (40% Formamide required) targeting yet uncultured phototrophic sulphur bacteria (Tonolla *et al.* 2005). After hybridization, the slides were transferred to prewarmed wash buffer [20mM Tris-HCl, 10mM EDTA, 0.01%SDS, either 450, 318, 159, 112, 80 or 56mM NaCl depending on Formamide concentrations (10, 20, 25, 30, 35 & 40% respectively)] incubated for 30min, and then rinsed with particle-free distilled water. Cells on the slides were counterstained with DAPI (4', 6-diamidino-2-phenylindole; final concentration, 0.2µg mL^{-1}) for 1 min, washed again, and dried. The cells were embedded in anti-fading mounting fluid (Vectashield, Vector laboratories Inc., Burlingame, CA) and viewed in an Olympus epifluorescent microscope (Olympus BX 51) equipped with a

monochromatic camera (Evolution VF cooled monochrome, Media cybernetics Inc, MD, USA). Filter sets NU, Cy3 and Cy5 were used for DAPI, Cy3 and Cy5 respectively. Images were processed by 'Image pro-express' software (Media Cybernetics Inc, MD, USA).

4.3. Results

4.3.1. Enrichment of photosynthetic sulphur bacterial consortia.

Enrichment under anaerobic condition in illuminated chambers was carried out with sediments and water samples collected from a variety of culture and marine environment. Based on the salinity regimes, cultures were developed for 5, 15, 25 and 40ppt. In total, 10 enrichment cultures of purple sulfur bacteria and 136 enrichment cultures of green sulfur bacteria could be developed. This collection of consortia formed the base for developing the bioremediation package for addressing the hydrogen sulphide toxicity in shrimp culture systems.

4.3.2. Screening of potential photosynthetic sulphur bacterial consortia.

Based on the sulfide removal rate, one consortium each from green and purple sulphur bacteria was segregated for salinities 5, 15, 25 and 40. The sulfide removal rates of selected bacterial consortia at the time of sampling were presented in Table 4.2. Four green sulphur bacteria and four purple sulphur bacteria were segregated from the collection of 146 enrichment cultures. On quantifying their potency in terms of millimole H_2S up take /Day/g biomass it varied from 0.0066 to 0.0192.

4.3.3. Enumeration of photosynthetic bacterial consortia

When the ultrasonicated samples were enumerated by epifluorescence microscopy, the highest number of cells obtained in the treatment was at 125W for 4min (Fig. 4.3). Ultrasonic treatments at 100W/1min., 100W/2min., 100W/3min., 125W/1min., and 125W/2min. were not satisfactory as biofilm were visible as clumps even after sonication.

4.3.4 Characterization of the segregated consortia

Out of the eight consortia segregated three (PSB 3, GSB 6 & GSB 7) consortia were segregated based on comparatively better growth observed during enrichment. They were subjected for the three level characterizations.

4.3.4.1 Transmission electron microscopy

Transmission electron micrograph of the consortium of purple sulphur bacteria (PSB-3) demonstrates aggregation of cells with stack like intracytoplasmic membranes, circular vesicles of cytomembranes, sulfur globule deposits, lamellar and tubular cytoplasmic membranes with inclusion bodies, numerous vesicles and gas vacuoles (Fig 4.4 a-i).

Consortium of green sulphur bacteria (GSB-6) demonstrated characteristic prostheca like outgrowth, prominent lamellar intracytoplasmic arrangements, internal and external sulfur granules with inclusion bodies and gas vacuoles embedded in thick slimy matrix. (Fig. 4.5 a-f)

Consortium of green sulphur bacteria (GSB-7) was characterized by extensive thick capsules with cells embedded. The capsulated cells were found covered by slime with clumps of cells aggregated. Besides, there are large vacuoles and prominent lamellar intra cytoplasmic arrangements. (Fig. 4.6. a-f).

4.3.4.2. Absorption spectra measurement

Bacteriochlorophylls have characteristic absorption maxima at specific wavelengths, bchl *a* (375, 590, 805, 830-890), bchl *b* (400, 605, 840, 1020-1040), bchl *c* (745-755), bchl *d* (725-745) and bchl *e* (710-725). Peaks obtained in the absorption spectra of PSB-3, GSB-6 and GSB-7 (Table 4.3 & Fig. 4.7) exhibited resemblance to bchl *a* and *b*.

4.3.4.3. Flourescent *in situ* hybridization

FISH analysis results are presented in Table 4.4. Autofluorescence was obtained but negligible. GSB-532, FISH probe for green sulphur bacteria hybridized with GSB 6 and 7 (Fig. 4.9 & 4.10) and not with PSB-3 (Fig. 4.8). Meanwhile, S-F-Chrom-986-b-A-20 hybridized with all the three consortia suggesting the presence of members of *Chromatiaceae* (Fig. 4.8, 4.9 and 4.10). Hybridization of S453F with GSB 6 and 7 suggested that the consortia do consist of yet uncultured photosynthetic sulphur bacteria (Fig. 4.9 and 4.10).

4.3.5. Mass culturing in photobioreactor

Two green sulphur bacterial consortia (GSB 6 & GSB 7) which were enriched in 25 and 15 salinity and one purple sulphur bacterial consortium (PSB 3) which could be

enriched in 15 salinity were amplified in the photobioreactor described. PSB 1, 2 & 4 were not maintaining their purple colour for longer duration on enrichment and were found taken over by green sulphur bacteria. GSB 5 & 8 exhibited poor growth in fermentor. Therefore with the three cultures sulphide removal rate was monitored and biomass harvested when sufficient growth was obtained. Biomass generated, rate of sulphide removal and yield coefficient determined were restricted to the three consortia (Table 4.5). The data generated suggested that there is low substrate up take but comparatively higher yield coefficient (17. 23 and 12. 55) with respect to the two green sulphur bacterial consortia such as GSB 6 & 7. However, PSB 3 exhibited comparatively lesser yield coefficient (2.98) and higher H_2S removal rate. Among the three consortia PSB 3 is the most efficient in terms of sulphide removal.

4.4. Discussion

To develop a broad base for an appropriate bioremediation of hydrogen sulphide toxicity, 146 enrichment cultures of photosynthetic sulphur bacteria were developed by simple enrichment technique. They were subjected to various levels of screening. At the first level screening four PSB consortia and four GSB consortia could be segregated for application in four salinity regimes such as 5, 15, 25 and 40. However, the H_2S up take potency of these cultures were found very low, primarily might be because of their growth conditions had not been standardized. However, with pure culture (*Chlorobium*), a removal rate of 0.87μmole/ L/ min/ mg protein has been recorded in a reactor with internal illumination (Lee and Kim, 1998). This suggested the need for standardization of culture conditions besides the requirement of purity, as presence of bacteria other than H_2S utilizers under anoxic conditions might retard the overall efficiency. This has to be a subject of further investigation.

A viable method for enumerating the consortia was standardized by ultra sonication and treatment at 125W for 4 minutes was found satisfactory. This has already been proved useful with respect to nitrifying bacterial consortia and has been proved else where also.

Even though 8 consortia could be segregated all of them were not found suitable for developing a bioremediation process by virtue of their slow growth rate. Therefore, from among the eight consortia one PSB consortium (PSB - 3) and two GSB consortia (GSB - 6 & 7) were subjected for further characterization as they were the cultures which showed active growth among the eight cultures segregated. Meanwhile, the other consortia

have also been maintained for further investigation in order to utilize them as possible candidates for bioaugmentation. It has to be appreciated that very little information could be gathered on their nutritional requirements and factors which would influence their growth potential. Since, this is a painfully time consuming process for immediate application three consortia were alone selected for further characterization and possible application.

The characterization was done at three levels such as, transmission electron microscopy, absorption spectra measurements and FISH analysis. Based on the cellular morphology, the consortia differed much in between and found distinct. Characteristic intracytoplasmic membranes, vesicles of cytomembranes, sulphur granules were seen in the PSB consortium (PSB – 3). In the GSB consortium (GSB – 6) prosthecae like growth, lamellar intrcytoplasmic membranes and internal and external sulphur granules were the characteristic features. In GSB - 7 extensive thick capsules with cells enclosed in it and cells embedded in slime matrix could be seen along with prominent intracytoplasmic membranes. Among these features deposition of sulphur granules and large sulphur pellets can be considered as the most pronounced identifying feature of their H_2S utilization potency. Similar observations have been made by Martinez-Alonso *et al.* (2006), while studying purple sulphur bacteria from marine microbial mat community. This property is most welcome trait for considering these consortia as bioaugmentors for bioremediation process as the sulphur removed from H_2S is deposited in the cell itself, paving the way for natural immobilization from the water column at least for a short time. Intracytoplasmic membranes observed in the TEM photomicrographs matches with the descriptions of the similar structures of the family *Rhodospirillaceae* and *Chromatiaceae* (Truper and Pfennig, 1981). The above evidences suggested that the consortia GSB 6 and 7 do consist of PSB. Due to the stringent light intensity requirements (near infra red range) PSB might not be getting enriched in the consortia paving the way for dominance of GSB, which had satisfied with a range of wavelength between 600-700nm. Apparently the overall observation dictates the requirements of modifying the light source and the specific wavelength to bring forth selective enrichment of PSB and GSB independently. This is an essential requirement for enhancing the efficacy of bioremediation as the light rays which reaches the bottom of the shrimp grow out are near to the infra red range as the shorter wavelength light rays are scavenged in the upper layers of water.

Bacteriochlorophylls measured based on absorbance exhibited resemblance to bchl *a* and *b*. This has been already established as characteristics of anoxygenic phototrophic

bacteria (Truper and Pfennig, 1981). FISH analysis of PSB – 3, demonstrated the presence of PSB alone in the consortium. On the other hand, GSB 6 & 7 contained both GSB and PSB suggesting them as mixed culture of both the types. Moreover, they consisted of yet uncultured photosynthetic sulphur bacteria also. This is an added evidence of existence of both PSB and GSB in the consortia named GSB - 6 and 7. The evidence of existence of yet uncultured PSB opens up avenues for further investigation using DGGE to investigate such organisms which are yet to be cultured.

Autoflourescence, generally reported in photosynthetic sulphur bacteria, is due to the liberation of bacteriochlorophylls by detergent like Triton X-100 generally used in certain fixatives and its subsequent conversion to bacteriopheophytin and related compounds (Tuschak, *et al.* 1999). However, in the present case Triton X-100 had not been included in the fixative and thereby the chances of autoflourescence were excluded.

On amplifying the consortia in a photobioreactor, GSB - 6 & 7 exhibited low substrate uptake and higher yield coefficient. But the above were better in PSB - 3 (higher substrate removal and low yield coefficient). The reason for better substrate uptake and low yield coefficient of PSB - 3 may be explained based on its fairly better purity in the sense that the consortium consisted of only PSB, not GSB as evidenced by ultra structural studies and FISH analysis. This is true in the sense that in GSB - 6 and 7 there is a strong evidence of both PSB and GSB, which diminishes their potency. There fore to transform these consortia for bioaugmentation, purification to separate the PSB and GSB as independent consortia/ organisms becomes an essentiality by selecting the most appropriate light waves by which these consortia can be re-enriched and on coupling with agar drop inoculation (Pfennig and Truper, 1981) the individual cells can be segregated for further cultivation.

The study unequivocally suggested that focus of further investigation must be on the purification of the consortia as either PSB or and GSB and go for single cell isolation so that pure culture bioaugmentors could be developed. Only with such cultures higher substrate uptake could be envisaged and there after the determination of their kinetics and development of their mass production technology will become fairly simpler.

Fig. 4.1: Photobioreactor set up.

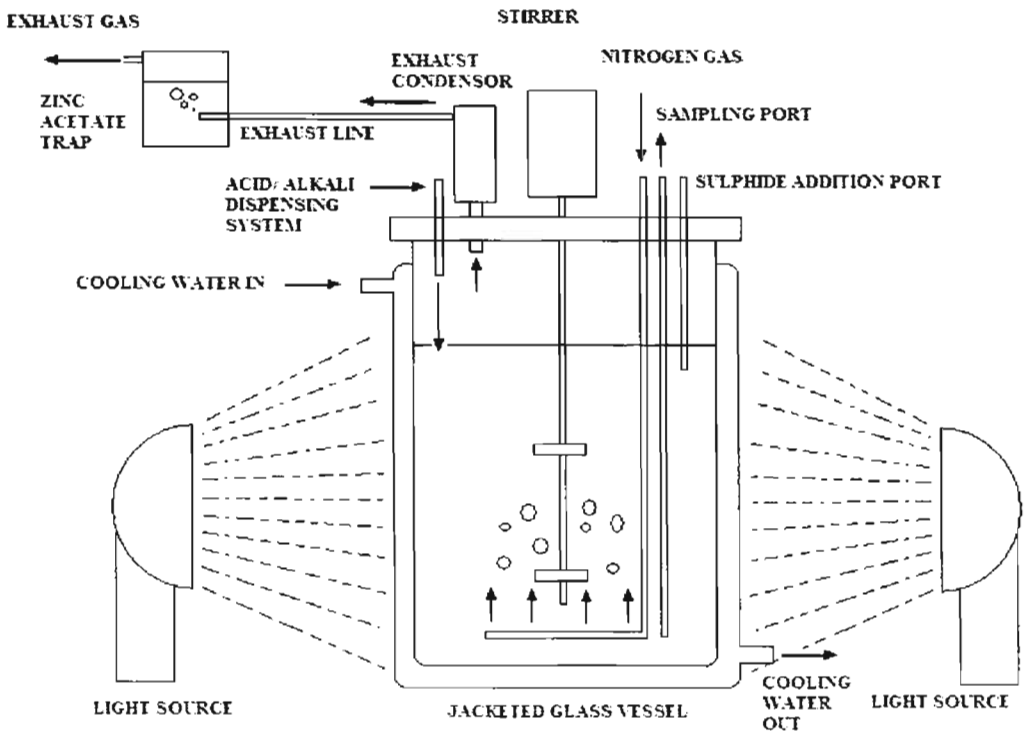
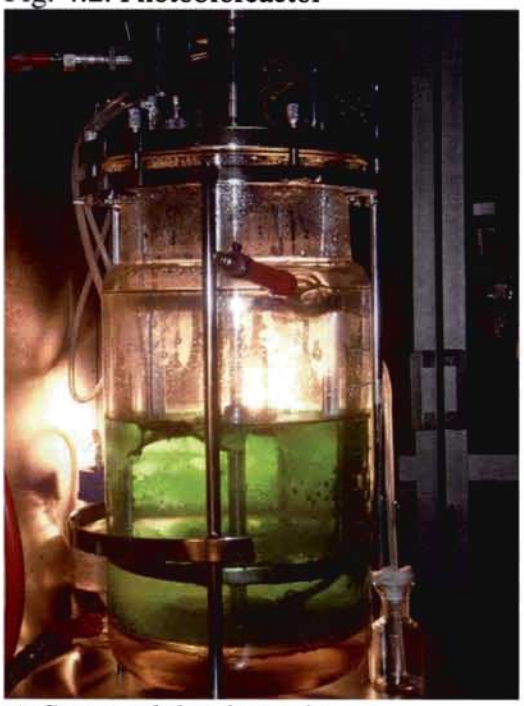


Fig. 4.2. Photobioreactor



a) Green sulphur bacteria

b) Purple sulphur bacteria

c) An overview of the experimental set up

Table 4.1: Details of FISH probes.

Name	Sequence 5'- 3'	Label	Target
GSB-532	TGCCACCCCTGTATC (15)	5' CY-3	Green sulphur bacteria.
S-F Chrom-986-b-A-20	TTCCRAGGATGTCAAGGGCT (20)	5' CY-3	Members of Chromatiaceae.
S453F	CCCTCATGGGTATTARCCACAAGGCG (26)	5' CY-5	Yet uncultured phototrophic sulphur bacteria.

Table 4.2: Hydrogen sulphide removal rate of selected cultures

Culture No.	Description of culture	H_2S removal rate (mmol H_2S/Day/g biomass)
1	Purple sulphur bacterial consortia enriched in 40ppt Sea water	0.0093
2	Purple sulphur bacterial consortia enriched in 25ppt Sea water	0.0066
3	Purple sulphur bacterial consortia enriched in 15ppt Sea water	0.0093
4	Purple sulphur bacterial consortia enriched in 5ppt Sea water	0.0123
5	Green sulphur bacterial consortia enriched in 40ppt Sea water	0.0129
6	Green sulphur bacterial consortia enriched in 25ppt Sea water	0.0096
7	Green sulphur bacterial consortia enriched in 15ppt Sea water	0.0093
8	Green sulphur bacterial consortia enriched in 5ppt Sea water	0.0192

Fig. 4.3 Number of cells obtained during different ultrasonic treatments (n=3)

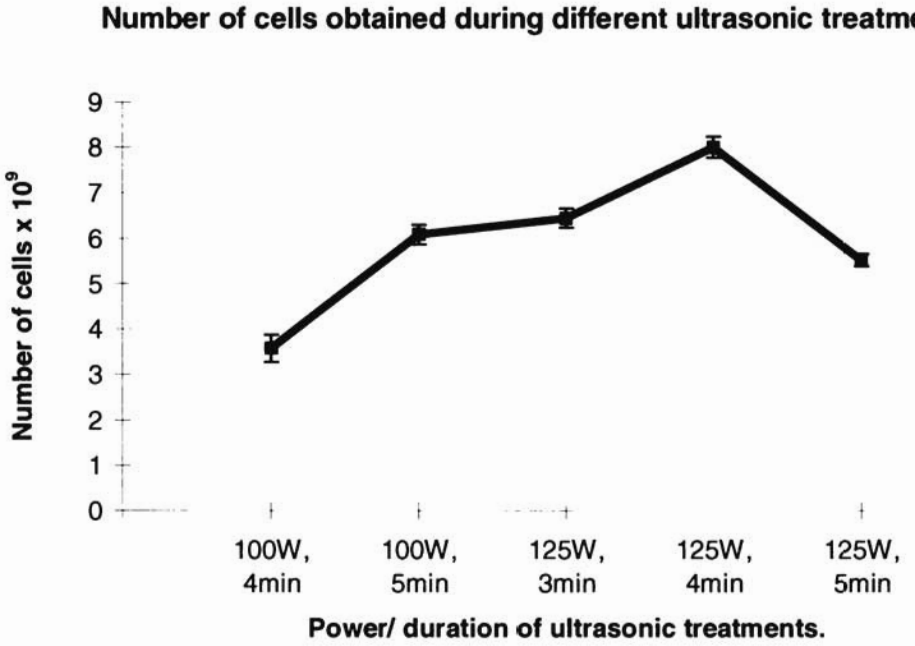
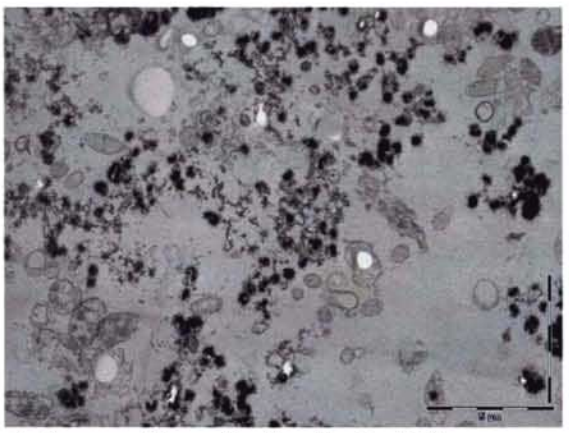
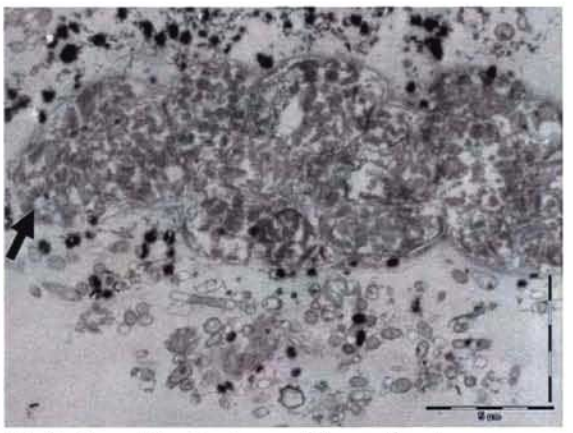


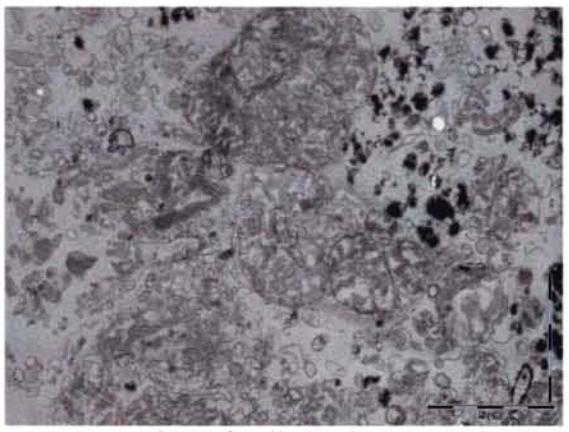
Fig. 4.4. Transmission Electron micrograph of PSB -3



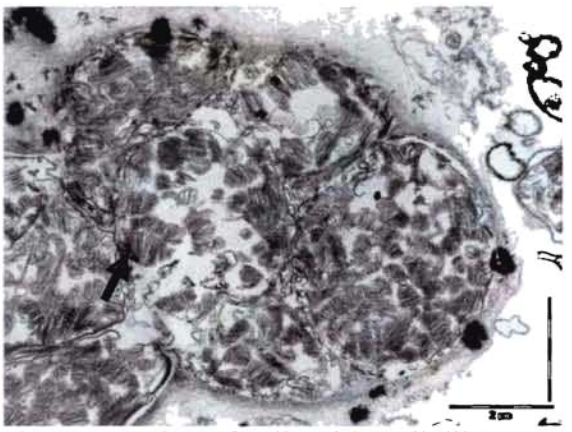
a) Aggregation of cells. Scale: 5.0μm



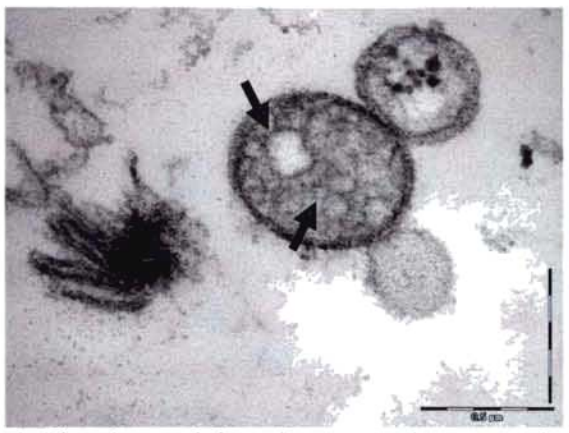
b) Cells with stacks and lamellar type cytomembranes. Scale: 5.0μm



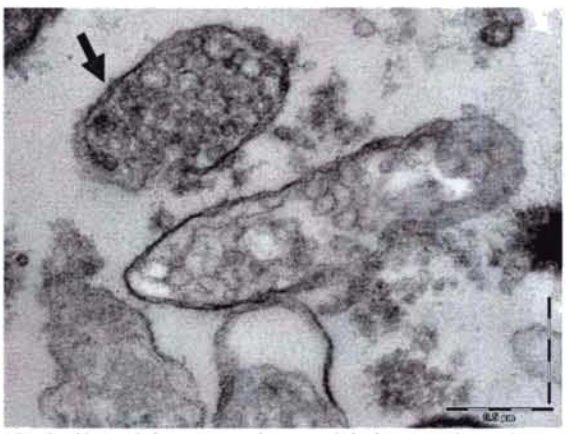
c) Aggregation of cells in the consortium Scale : 5.0μm.



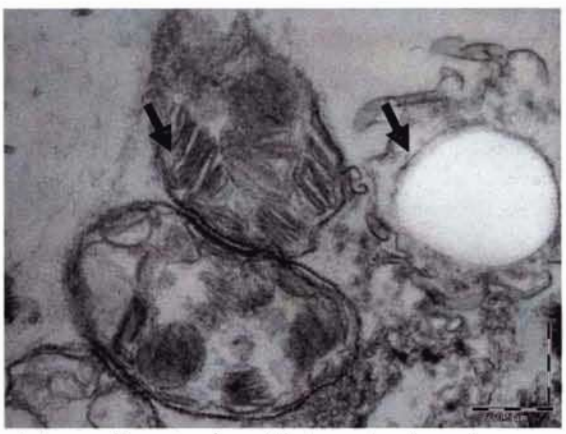
d) Aggregation of cells with stalk like intracytoplasmic membranes.Scale: 2.0μm



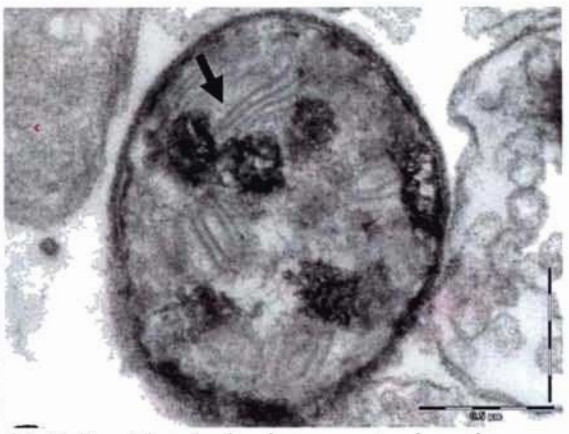
e) Circular vesicles of cytomembranes and sulphur globules. Scale: 0.5μm



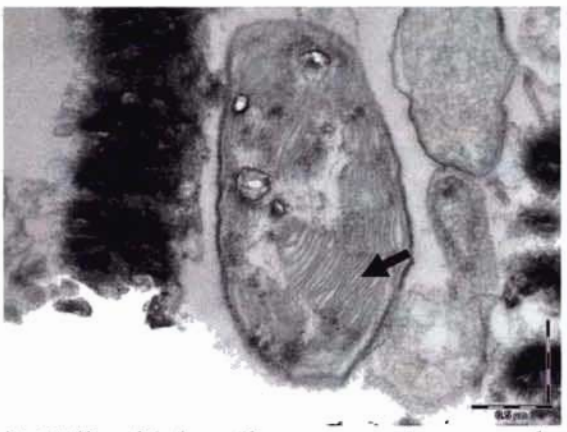
f) Cells with extensive vehicles and and gas vacuoles. Scale 0.5μm



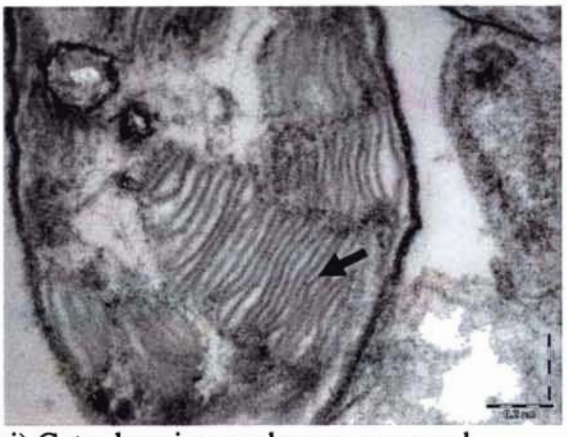
g) Lamellar stacks of intracytoplasmic membrane system and larger sulphur deposit. Scale 0.5μm



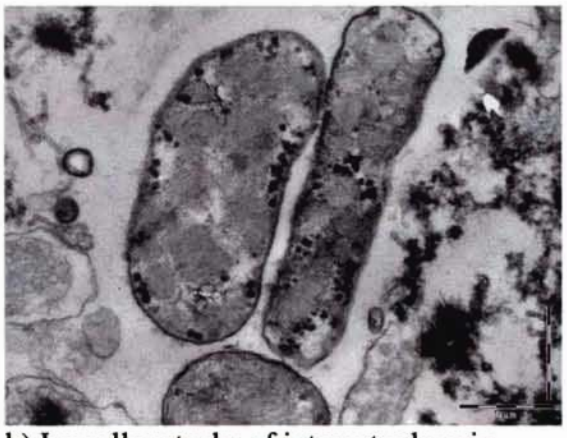
h) Cell with tubular intra- cytoplasmic membranes. Scale 0.5μm



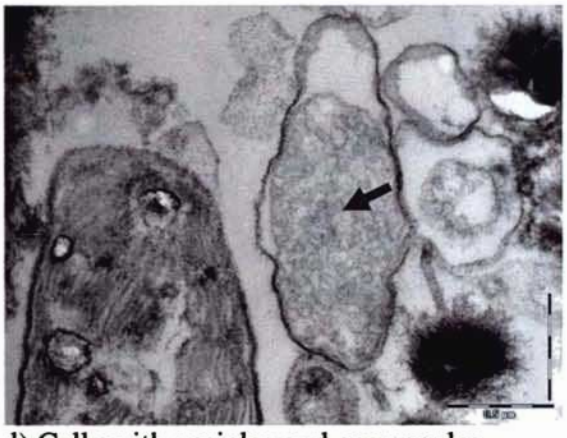
i) Cells with lamellar cytomembranes and sulphur granules. Scale 0.5μm.



j) Cytoplasmic membranes arranged as lamellae. Scale 0.2μm

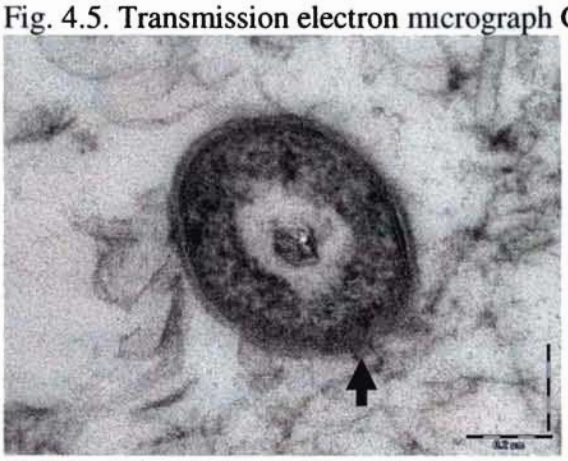


k) Lamellar stacks of intracytoplasmic membranes. Scale: 1.0μm

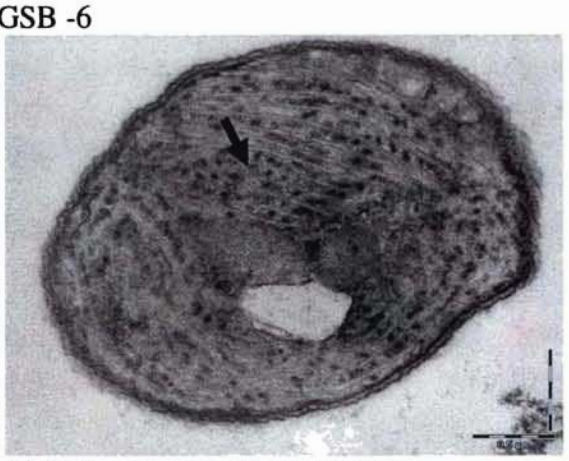


l) Cells with vesicles and gas vacules. Scale :0.5μm

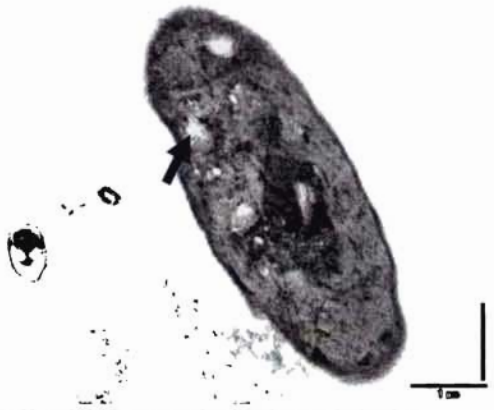
Fig. 4.5. Transmission electron micrograph GSB -6



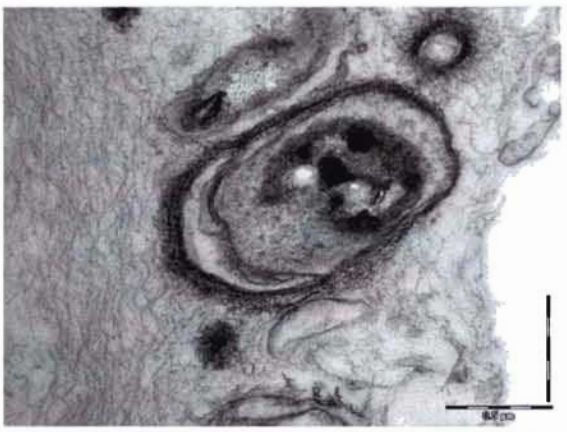
a) Cell with prosthecae like out growth Scale 0.2µm.



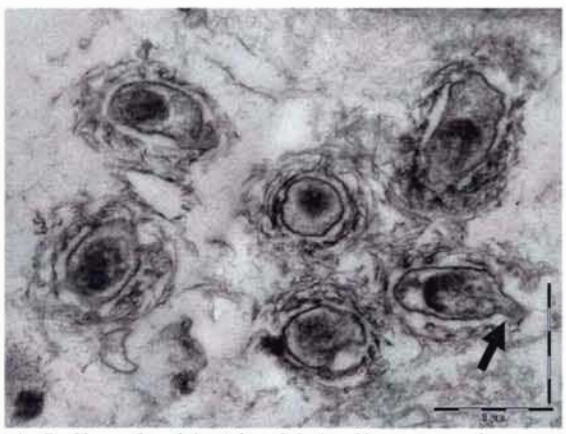
b) Cell with prominent lamellar intracytoplasmic arrangement. Scale 0.5µm



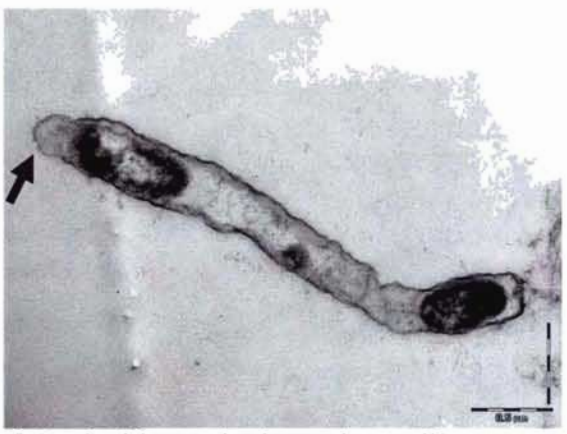
c) Cell with internal sulphur globules. Scale 1.0µm.



d) Cell with inclusion bodies, gas vacuoles and sulphur globules. Scale 0.5µm

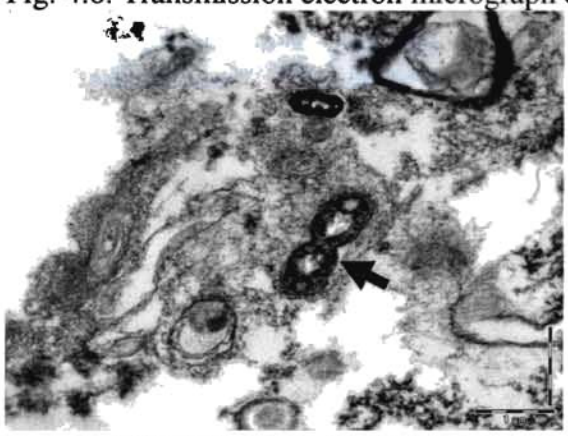


e) Cell embedded in thick slimy matrix with prosthecae like structure. Scale 1.0µm.

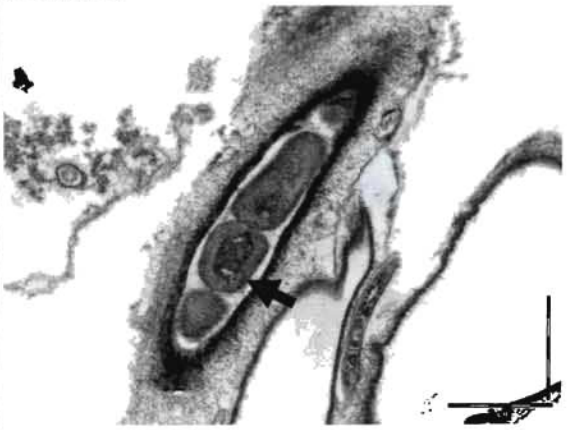


f) Rod with terminal prosthecae like structure. Scale 0.5µm

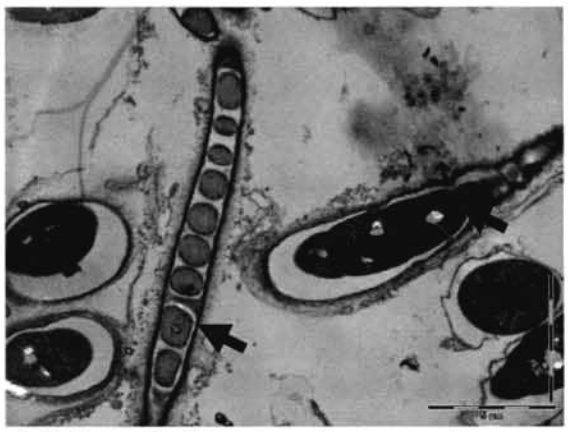
Fig. 4.6. Transmission electron micrograph of GSB -7



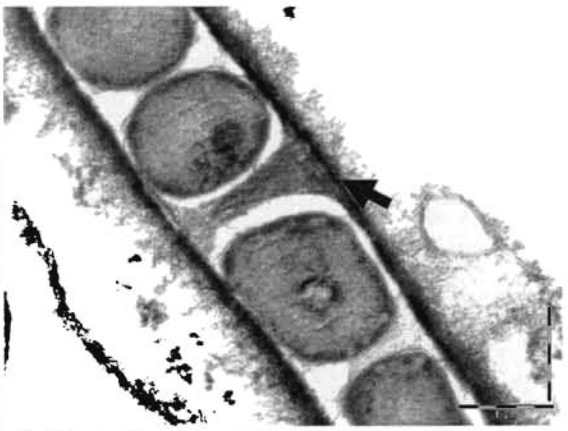
a) Consortium: Budding. Scale 1.0μm



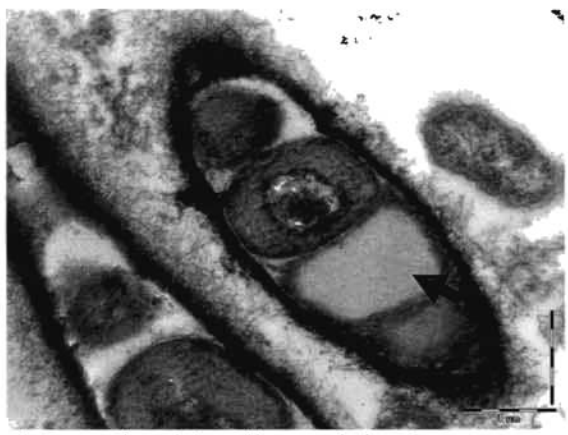
b) Cells embedded in capsules. Scale 2.0μm



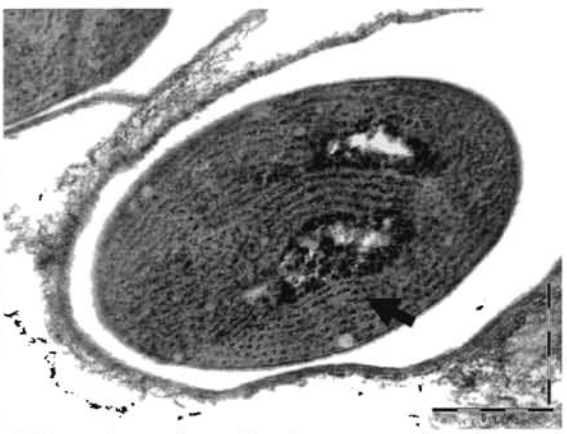
c) Cells embedded in a slime capsule, clumps of cells seen as aggregates. Scale 5.0μm



d) Capsule with cells. Scale 1.0μm



e) Capsule with cell containing large gas vacuole. Scale 1.0μm



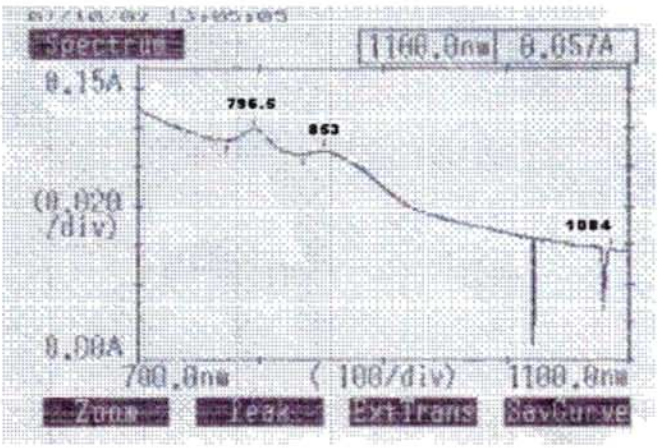
f) Prominent lamellar intracytoplasmic arrangements. Scale 1.0μm

Table 4.3: Details of absorption spectra measurement of selected cultures.

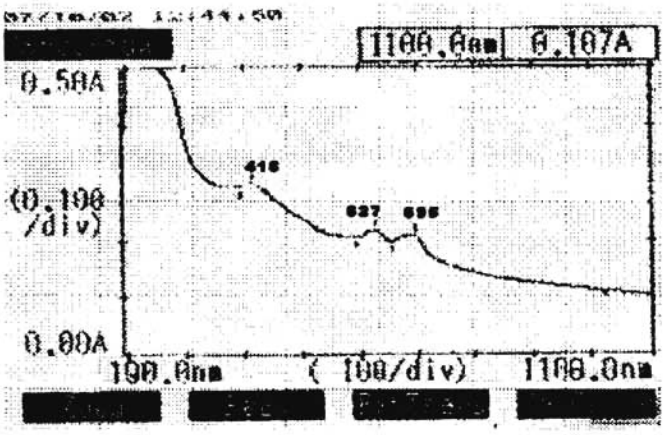
Consortium.	Peaks obtained	Characteristic absorption maxima obtained (nm)	Bacteriochlorophyll Type present.
3	853 (830-860) 796.5 (780-805)	830-890 805	bchl *a*
6	868 (820-890) 681 (650-690) 621 (580-630)	830-890, 840 590, 605	bchl *a* and *b*
7	695 (650-700) 627 (600-630) 416 (400-430) 380	 605 400 375	bchl *b*

Fig.4.7. Absorption spectra of photosynthetic sulphur bacterial consortia

PSB – 3



GSB- 7



GSB - 6

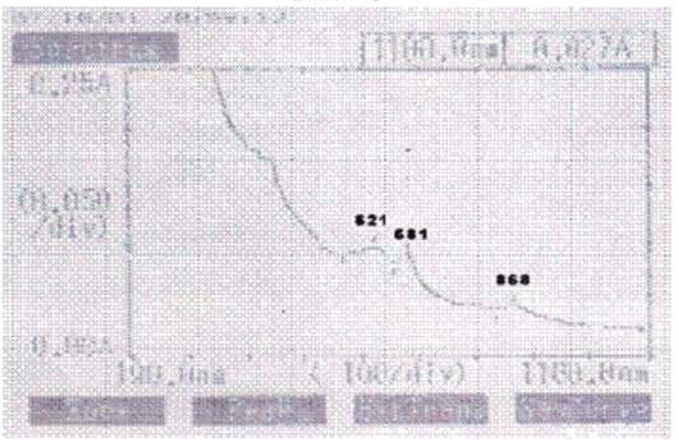
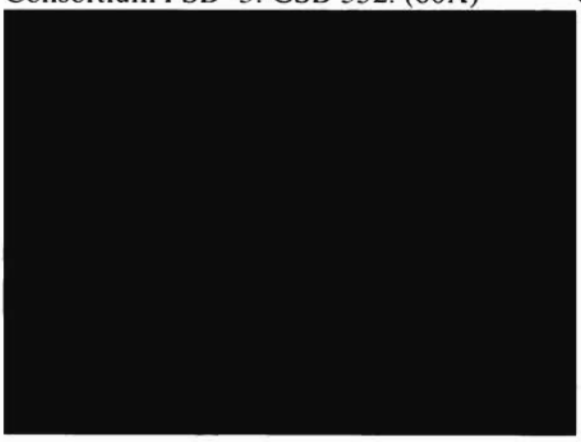


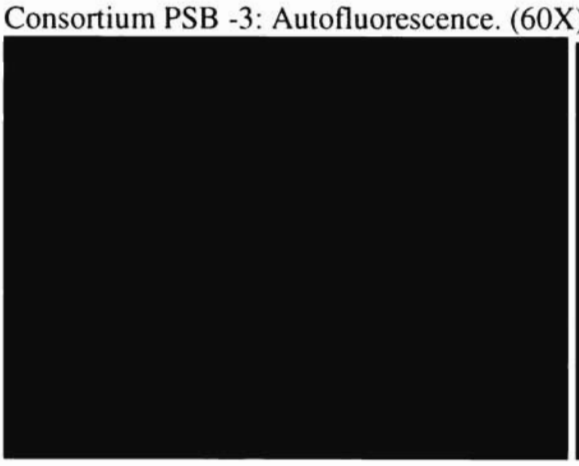
Fig.4.8. Fluorescent *in situ* hybridization of purple sulphur bacterial consortium (3).

Consortium PSB -3: GSB 532. (60X)

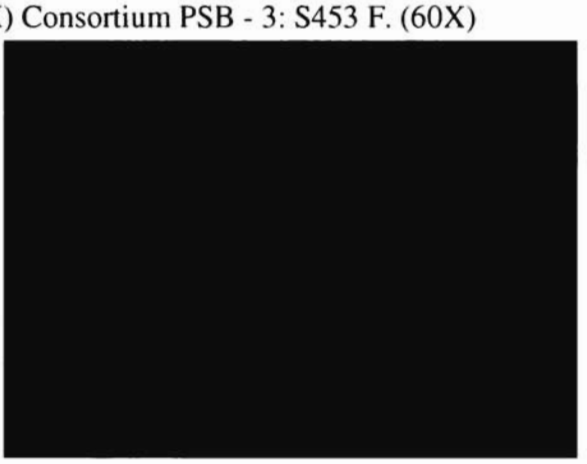


Consortium PSB -3: DAPI. (60X)

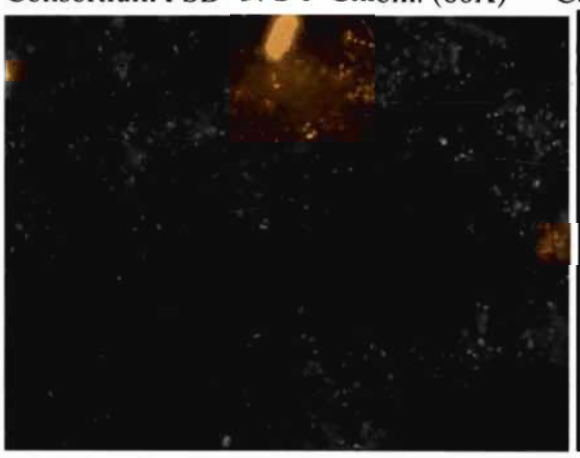
Consortium PSB -3: Autofluorescence. (60X)



Consortium PSB - 3: S453 F. (60X)



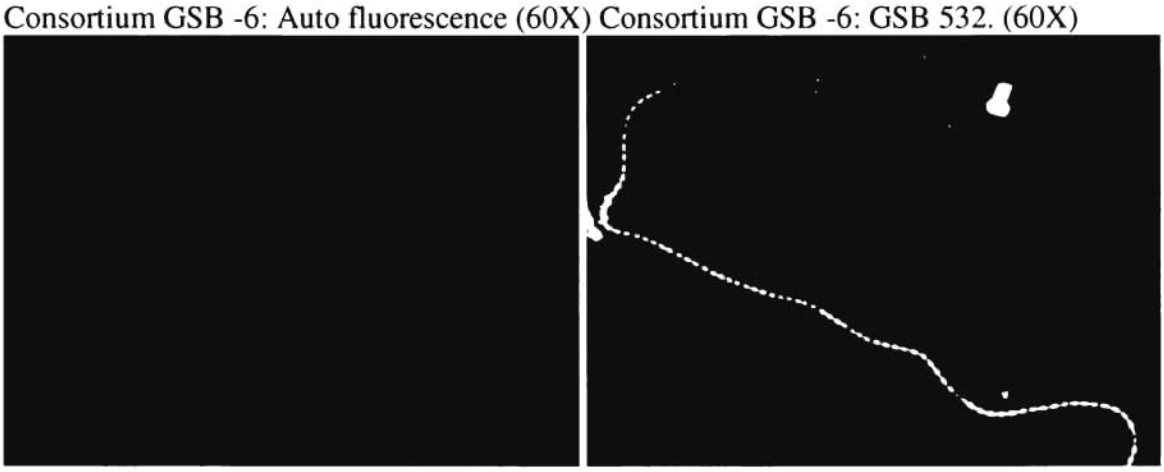
Consortium PSB -3: S-F-Chrom. (60X)



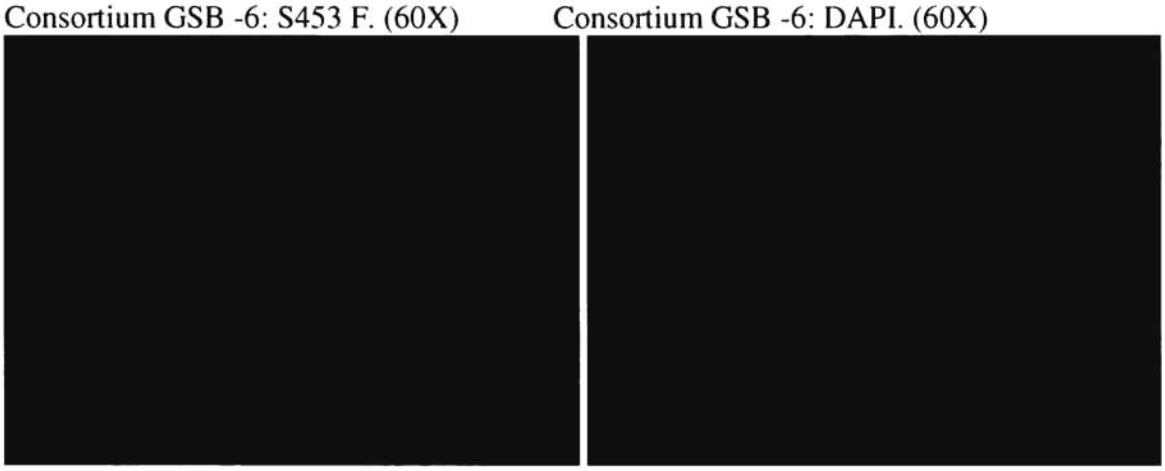
Consortium PSB -3: DAPI. (60X)

Fig.4.9. Fluorescent *in situ* hybridization - Green sulphur bacterial consortium GSB - 6.

Consortium GSB -6: Auto fluorescence (60X) Consortium GSB -6: GSB 532. (60X)



Consortium GSB -6: S453 F. (60X) Consortium GSB -6: DAPI. (60X)



Consortium GSB - 6: S-F Chrom. (60X) Consortium GSB 6: DAPI. (60X)

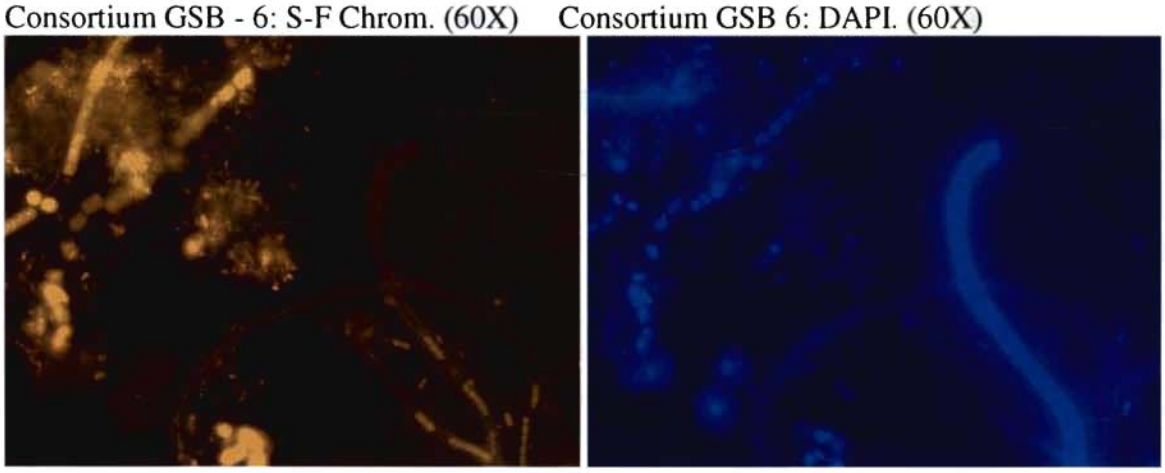
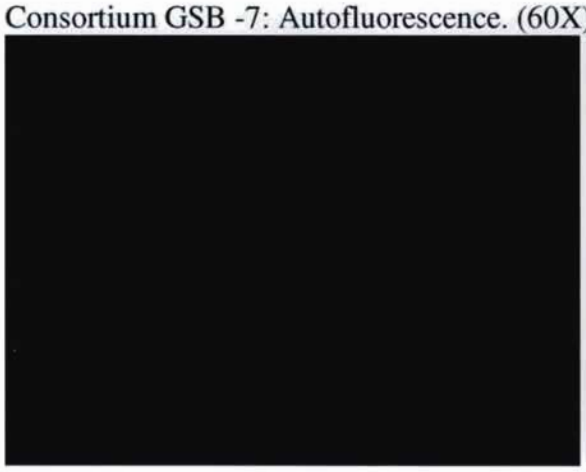
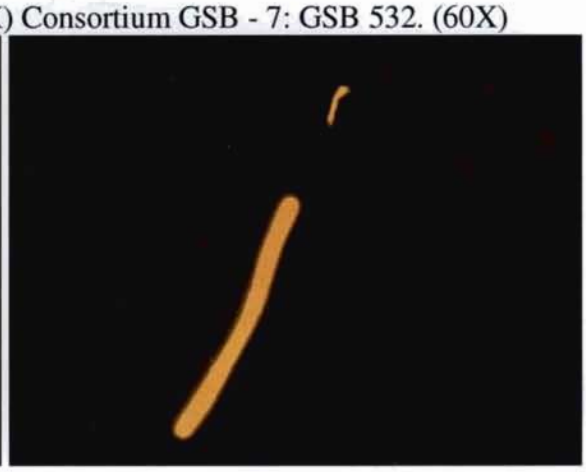


Fig.4.10. Fluorescent *in situ* hybridization - green sulphur bacterial consortium (7)

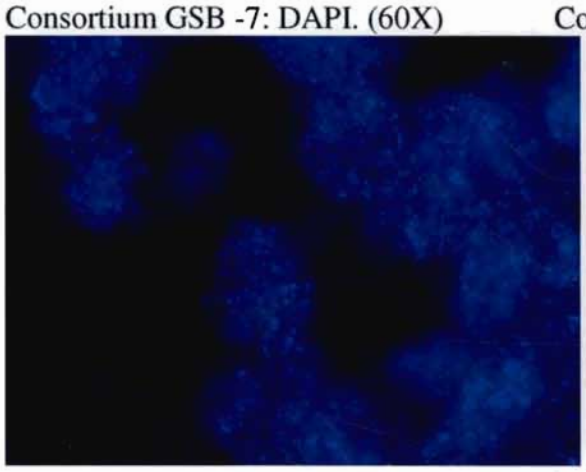
Consortium GSB -7: Autofluorescence. (60X)



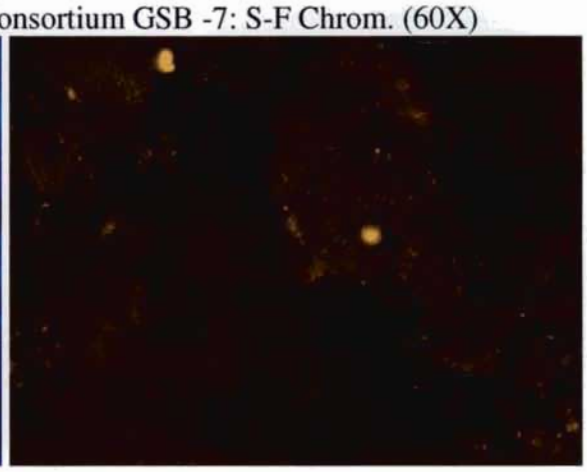
Consortium GSB - 7: GSB 532. (60X)



Consortium GSB -7: DAPI. (60X)



Consortium GSB -7: S-F Chrom. (60X)



Consortium GSB -7: S453 F. (60X)

Consortium GSB -7: S453 F. (60X).

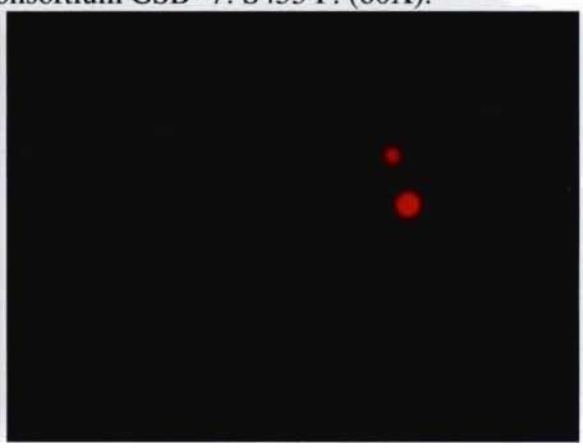


Table 4.4: FISH probe binding in selected consortia.

Consortium	Autoflourescence	GSB-532	S-F-Chrom-986-b-A-20.	S453F
3	Nil	Negative	Positive	Negative
6	Slight	Positive	Positive	Positive
7	Slight	Positive	Positive	Positive

Table: 4. 5. Details of sulphide removal obtained in photobioreactor.

Consortium	Specifications	Total biomass generated	Culture period	Sulphide removal rate (mM H_2S/day/g biomass)	Yield Coeffecient (Mass of cells generated (mg/L)/Mass of substrate utilized (md/L)
PSB 3	Purple sulphur bacterial consortia enriched in 15ppt Sea water	0.873g	25 days	0.958	2.98
GSB 6	Green sulphur bacterial consortia enriched in 25ppt Sea water	4.576g	90 days	0.053	12.55
GSB 7	Green sulphur bacterial consortia enriched in 15ppt sea water	3.842g	24 days	0.194	17.23

Chapter 5

Conclusions and future research needs

5.1 Conclusions

Shrimp grow out systems are specialized and highly dynamic aquaculture production units which when operated under zero exchange mode require bioremediation of ammonia, nitrite nitrogen and hydrogen sulphide to protect the crop. The research conducted here is to develop an economically viable and user friendly technology for addressing the above problem. The nitrifying bacterial consortia (NBC) generated earlier (Achuthan *et al.*, 2006) were used for developing the technology. For addressing hydrogen sulphide toxicity, consortia of photosynthetic sulphur bacteria have been generated in this programme itself. This chapter deals with the synthesis of information generated and the technology developed for addressing these two main concerns in zero water exchange shrimp culture systems.

For the development of bioaugmentation of ammonia toxicity, the NBC were subjected for characterization, for which the consortia maintained at 4°C were amplified in a fermentor initially. While doing so, they were found to perform denitrification also. This has been suggested as an ideal situation as far as the aquaculture systems are concerned, as removal of ammonia to dinitrogen is the process highly favourable for the cultured stock.

The consortia were purified on phytagel plates. Accordingly, a total of 96 pure cultures were generated from all four consortia all together. All the isolates exhibited heterotrophic growth. Previous literature suggests that some but not all nitrite oxidizers can grow mixotrophically in a medium supplemented with yeast extract and peptone as nitrogen sources and pyruvate or acetate as the carbon source. Majority of the pure cultures obtained from the NBC in the study were Gram positive, either rods or cocci, with actinomycetes like colony morphology. Out of 96 isolates only 25 were Gram negative. As on date, Gram positive autotrophic nitrifiers have not been reported. However, in the consortia under investigation they were the dominant forms. According to literature, most but not all nitrifiers have a typical Gram negative multilayered cell wall. However, the cultures resolved from NBC which exhibited heterotrophic growth can not be nomenclatured as heterotrophic nitrifiers as they generate nitrite, the unique product of autotrophic nitrification.

Considering the heterotrophic growth, all the isolates were subjected for biochemical characterization and clustering based on unweighted average linkage. Several distinct clusters (42) having 80% and above similarity could be differentiated. This treatment helped in selecting representatives for molecular level analysis of the genes

encoding *amoA, nirS* and *nirK*. However, with the phenotypic characteristics investigated the clusters could not be identified.

On examining the ultra structural details of the consortia and cells, characteristic features of nitrifiers such as intracytoplasmic membranes, carboxysomes, polyphosphate like inclusions, EPS, cysts, zoogloea, curved rods, etc could be demonstrated. All these characteristics have been documented as unique features of autotrophic nitrifiers. This observation assumes very important dimensions in the light of evidence of heterotrophic growth of the isolates; therefore, a judgment on their status as either heterotrophic nitrifiers or autotrophic nitrifiers has to be made based on these evidences.

All the 96 pure isolates from the consortia exhibited nitrifying potency, when they were incubated under obscurity with acration and agitation. The requirements of aeration and agitation have been observed with the consortia for efficient nitrification through out the experiment. This can be taken for granted as a specific requirement for nitrification as mass transfer is the key factor for efficient oxidative processes in general.

One of the most interesting and important observations with respect to the pure cultures resolved from the consortia is their denitrifying potency, exhibited as nitrate reduction and/ or nitrite reduction. Among them 47 isolates exhibited both the properties. Both nitrifying and denitrifying properties were exhibited by 90 isolates which could be considered as the most unique feature of the consortia as the organisms in them were able to convert ammonia nitrogen or nitrite nitrogen to the products of denitrification subsequent to nitrification. This is a complex situation not reported earlier. No bacterial species which oxidizes ammonia to nitrate directly has been found to date. However, possibility of such direct conversions has been postulated by a few workers.

In support to the above, efforts were made to amplify *amoA* gene for ammonia oxidation and *nirS* and *nirK* genes for nitrite reduction. In several of the isolates these genes could be amplified even though there were band shifts. Failure to amplify these genes in other isolates which expressed the nitrifying and denitrifying potency phenotypically, does not suggest their absence, as much more standardization of the PCR reactions have to be carried out before coming to a conclusion.

With these consortia, efforts were made to develop a user friendly product which can be applied for bioremediation of ammonia toxicity at times of need. As these consortia formed biofilm, it was decided to develop a simple, less expensive, environment friendly delivery system for application. As biodegradable carriers are required for the above which will not leave any residue on degradation, wood powder from four woody

plants common in the region, and chitin from prawn shell waste were chosen for screening and selecting the best. However, the main concern in this work has been to identify an appropriate carrier material for application in shrimp grow out systems.

A protocol for delignification was standardized as lignin was thought to interfere with immobilization. According to the protocol, treatment of wood powder with 1% (v/v) H_2O_2 in tap water for a period of 3-5 hours at pH 11.5 was found sufficient to oxidize lignin to the point of extinction. In the practical sense the methodology standardized was simple and comparatively less expensive.

The mode of immobilization on wood powder and chitin as the substrata has been adsorption, proved as the best method of choice for immobilizing nitrifying bacteria.

For the soundness of the technology it was decided to screen and select the most appropriate substratum from among the five categories of the materials short listed for the development of the technology. The first round of screening of the substratum was on the basis of surface profile and accordingly delignified *A. altissima* (300-500µm and 500-710µm) particles exhibited the largest number of corrugations indicating more surface area for attachment. In the second round of screening, two dimensional surface areas of the samples by image analysis of scanning electron micrographs was accomplished and the particles of *A. altissima* was found to have the highest surface area. Through BET analysis specific surface area of the substratum was determined as 1.87 m^2/g. Being biodegradable, after a period of time, the substratum shall get disintegrated leaving no residue.

It was essential to confirm the primary selection made here of the substratum by further rounds of screening based on preference of the NBC in terms of adsorption on to the particles and their nitrification potential. To facilitate the above, activation of the NBC stored at 4°C could be accomplished by inoculating and incubating 10mL consortia in Watson's medium under aeration in obscurity.

For all practical purposes enumeration of cells in NBC was a challenge, which was addressed by ultrasonication at 125W for 4 minutes.

On experimenting with the dissociated cells in all the five category of the substrata *A. altissima* with particle size 300-500 and 500-710µm was found to support uniformly all NBC in terms of least coefficient of variance (Cv). Subsequently, by way of preferential attachment of the NBC the substratum derived from *A. altissima* with particle size 300-1500µm was identified and confirmed for all further immobilization purposes.

Time course of immobilization of NBC was investigated and within 24 hours itself biofilm formation was found much progressed under optimum conditions. Prolonged incubation for immobilization was leading to disintegration of the substratum.

Experiments to determine the least number of cells required for immobilizing in unit weight / area of the substratum suggested that 10^5 to 10^8 cells per mL in 100mL medium did not make any significant differences for immobilization on 1g substratum. This led to the realization that the lowest count of 10^5 cells per mL would be sufficient to get the cells attached satisfactory on 1g substratum per 100mL medium. This suggest that even though the cell count during immobilization happens to be lower comparatively, on adsorption and colonization they can grow rapidly forming biofilm and perform nitrification at the same rate of the immobilized substratum with initial higher number of bacterial cells.

For economic viability minimum quantity of the immobilized NBC required for treating unit volume of sea water was determined and found that with in the range 0.2 to 1.0g immobilized NBC there was progressive increase of nitrification in 20L Seawater at 15 and 30 salinity suggesting that the quantity of immobilized NBC was directly correlated with activity. However, the nitrification per se with in the range tested was not significantly different. This offered much flexibility to the choice in the quantity of the immobilized NBC for application. This is a clear demonstration of better quality of immobilized nitrifiers generated in this study for field application.

In all commercial applications of the immobilized NBC, shelf life happens to be a major issue to be sorted out. In this case, storage of the immobilized nitrifiers for a month under ambient conditions did not affect much the nitrifying potency; however, TAN removal rates were slightly reduced. This is an after effect of long term storage which has generally been experienced both with pure cultures and consortia. But when they are brought to a fresh medium and incubated under aeration and agitation the activity have been found regaining. This suggests that before application, the immobilized nitrifiers have to be aerated and agitated to perform better.

Surprisingly the NBC were found with denitrification potency. On examining the same under anaerobic conditions, NO_3^--N removal was well correlated with NO_2^--N suggesting the existence of dissimilatory denitrifiers. This observation was quiet true in the sense that the pure cultures resolved from the consortia exhibited denitrifying potency. This situation is advantageous in the sense that a single product on application can perform both nitrification and denitrification.

On evaluating the nitrifying potency of immobilized NBC in a simulated bioassay system the extend of nitrification was double the one recorded in control suggesting their probable efficacy under field conditions at salinities 10, 20 and 32. This experiment proves the usefulness of the product on mitigating ammonia toxicity in aquaculture systems.

Subsequently a simple device was designed and fabricated for mass immobilization of NBC. A three day treatment in this system was found satisfactory to effectively immobilize the nitrifiers for field application. Further incubation did not add to the efficacy. The device is simple and user friendly and can be manufactured and operated with less capital investment and operational cost. However, more studies have to be conducted on the kinetics of immobilization and the shelf life of the product generated from this system. A point of concern is the involvement and interference of natural microbial flora present in the seawater used for suspending the bacterial consortium and the substratum for immobilization. According to the protocol which has been experimented the sea water was not sterilized by any means. An extensive and thorough analysis of this situation is most warranted.

One of the consortia (AMOPCU-1) on applying in a shrimp grow out system, 50% lowering of TAN was recorded. Even though from this single experiment it may not be possible to conclude the efficacy of the immobilized NBC in bioremediating ammonia toxicity in shrimp grow out systems. The data generated suggest that by repeated trial and error method an efficient bioremediation package for ammonia toxicity for shrimp grow out systems can be brought out with the products which have been generated in this study.

To develop a broad base for an appropriate bioremediation of hydrogen sulphide toxicity, 146 enrichment cultures of photosynthetic sulphur bacteria were developed by simple enrichment technique. They were subjected for various levels of screening. At the first level screening four purple sulphur bacterial (PSB) consortia and four green sulphur bacteria (GSB) consortia could be segregated for application in four salinity regimes such as 5, 15, 25 and 40. However, the H_2S up take potency of these cultures were found very low, primarily might be because of their growth conditions had not been standardized. This suggests the need for standardization of culture conditions besides the requirement of purity, as presence of bacteria other than H_2S utilizers under anoxic conditions might retard the overall efficiency. This has to be a subject of further investigation.

A viable method for enumerating the consortia was standardized by ultra sonication and treatment at 125W for 4 minutes was found satisfactory. This has already

been proved useful with respect to nitrifying bacterial consortia and has been proved else where also.

Even though 8 photosynthetic sulphur bacterial consortia could be segregated, all of them were not found suitable for developing a bioremediation process by virtue of their slow growth rate. Therefore, from among the eight consortia one PSB consortium (PSB - 3) and two GSB consortia (GSB - 6 & 7) were subjected for further characterization as they were the cultures which showed comparatively better active growth among the eight cultures segregated. Mean while the other consortia has also been maintained for further investigation in order to utilize them as possible candidates for bioaugmentation. It has to be appreciated that very little information could be gathered on their nutritional requirements and factors which would influence their growth potential. Since, this is a painfully time consuming process for immediate application three consortia were alone selected for further characterization and possible application.

The characterization was done at three levels such as transmission electron microscopy, absorption spectra measurements and FISH analysis. Based on the cellular morphology, the consortia were found differing much in between and found distinct. Characteristic intracytoplasmic membranes, vesicles of cytomembranes, sulphur granules were seen in the PSB consortium (PSB – 3). In the GSB consortium (GSB – 6) prosthecae like growth, lamellar intracytoplasmic membranes and internal and external sulphur granules were the characteristic features. In GSB 7 extensive thick capsules with cells enclosed and slime in which cells embedded could be seen along with prominent intracytoplasmic membranes. Among these features, deposition of sulphur granules and large sulphur pellets can be considered as the most pronounced identifying feature of their H_2S utilization potency. This property is a most welcome trait for considering these consortia as bioaugmentors for bioremediation process as the sulphur removed from H_2S is deposited in the cell itself, paving the way for natural immobilization from the water column at least for a short time. Intracytoplasmic membranes observed in the TEM photomicrographs match with the descriptions of the similar structures of the family *Rhodospirillaceae* and *Chromatiaceae*. The above evidences suggest that the consortia GSB 6 and 7 do consist of PSB. Due to the stringent light intensity requirements (near infra red range) PSB might not be getting enriched in the consortia paving the way for dominance of GSB, which had satisfied with a range of wavelength between 600-700nm. Apparently the overall observation dictates the requirements of modifying the light source and the specific wavelength to bring forth selective enrichment of PSB and GSB

independently. This is an essential requirement for enhancing the efficacy of bioremediation as the light rays which reaches the bottom of the shrimp grow out are near to the infra red range as the shorter wavelength light rays are scavenged in the upper layers of water.

Bacteriochlorophylls measured based on absorbance exhibited resemblance to bchl *a* and *b*. This has been already established as characteristics of anoxygenic phototrophic bacteria. On FISH analysis, PSB - 3 demonstrated the presence of PSB alone in the consortium. On the other hand, GSB 6 & 7 contained both GSB and PSB suggesting them as mixed culture of both the types. Moreover they consisted of yet uncultured photosynthetic sulphur bacteria also. This is an added evidence of existence of both PSB and GSB in the consortia named GSB - 6 and 7. The evidence of existence of yet uncultured photosynthetic sulphur bacteria opens up avenues for further investigation using DGGE to investigate such organisms which are yet to be cultured.

On amplifying the consortia in a photobioreactor, GSB - 6 & 7 exhibited low substrate uptake and higher yield coefficient. But the above were better in PSB - 3 (higher substrate removal and low yield coefficient). The reasons for better substrate uptake and low yield coefficient of PSB - 3 may be explained based on its fairly better purity in the sense that the consortium consisted of only PSB, not GSB as evidenced by ultrastructural studies and FISH analysis. This is true in the sense that in GSB - 6 and 7 there is a strong evidence of both PSB and GSB, which diminishes their potency.

5.2 Future Research Needs

1. Four nitrifying bacterial consortia have been partially characterized. While doing so it has been realized that the bacterial diversity in them is so high and it needs molecular taxonomic approaches to delineate the species involved. The situation is that, the same consortium consists of nitrifying and denitrifying bacteria together and also having the functional genes such as *amoA, nirS* and *nirK* also in one organism makes them an entity worth investigating further. All pure cultures need to be identified based on 16S rRNA gene sequence analysis. PCR amplification of *amoA, nirS* and *nirK* genes have to be standardized and the amplicons have to be essentially sequenced.

2. Technology for immobilizing nitrifying bacterial consortia on wood powder as the substratum has been standardized. However, the mass production has to be further

optimized and scaled up for initiating commercial production. Issues like interference from natural bacterial community during immobilization have to be sorted out.

3. Extensive field level application and validation of the immobilized consortia have to be accomplished and based on the results an application protocol can be standardized.

4. The photosynthetic sulphur bacteria enriched (four purple sulphur bacteria and four green sulphur bacteria) needs further study in terms of purification subsequent to selective enrichment under near infrared and at around 600 to 700 nm coupled with agar drop inoculation. It has been realized that pure culturcs of photosynthetic sulphur bacteria shall perform better than consortia and all efforts should be made to purify them and to determine their potency for selecting the best strain.

5. An appropriate photo bioreactor with internal illumination is essential as, the one with external light source enhances wall growth and diminishes the prospects of larger biomass generation.

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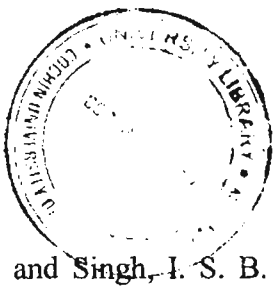

Appendix

Publication and patents arising out of this thesis

Publications.

Manju, N. J., Thomas, A., Kumar, V. J. R., Pai, S. S., Philip, R. and Singh, I. S. B. Resolution of putative Nitrifying bioaugmentors, for application in aquaculture. (Manuscript).

Manju, N. J., Deepesh, V., Philip, R. and Singh, I. S. B. Immobilization of nitrifying consortia on wood powder for application in shrimp culture systems. (Manuscript).

Manju, N. J., Deepesh, V., Philip, R. and Singh, I. S. B. Enrichment cultures of photosynthetic sulphur bacteria for oxidation of dissolved hydrogen sulphide from aquaculture systems. (Manuscript).

Related publications

Singh, I. S. B., Achuthan, C., Kumar, V. J. R., **Manju, N. J.** (2003) Bioreactor technology for rapid removal of ammonia and nitrite from prawn larval rearing systems. In Aquaculture Medicine (Eds) Singh, I. S. B., Pai, S. S., Philip, R. and Mohandas, A. CFDDM, School of Environmental Studies, CUSAT. ISBN 81-900724-1-2. pp 133-134.

Achuthan, C., Kumar, V. J. R., **Manju, N. J.**, Philip, R. and Singh, I. S. B. (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.

Patent

Patent application process in progress.

Title of invention:

Immobilization of nitrifying bacterial consortia on wood powder as carrier material and mass production. Singh, I. S. B., **Manju, N. J.**, Deepesh, V. and Philip, R.