DEVELOPMENT OF BIOREACTORS FOR NITRIFYING WATER IN CLOSED SYSTEM HATCHERIES OF PENAEID AND NON-PENAEID PRAWNS

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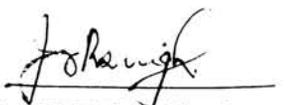
Certificate

This is to certify that the research work presented in this thesis entitled **'Development of bioreactors for nitrifying water in closed system hatcheries of penaeid and non-penaeid prawns'** is based on the original work done by Ms. Cini Achuthan under my guidance, in the School of Environmental Studies, Cochin University of Science and Technology, Cochin 682 016, in partial fulfilment 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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GENERAL INTRODUCTION

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1.1. Nitrification

Nitrification is the biological oxidation of reduced forms of nitrogen, usually ammonium, to nitrate. The group of bacteria of the family Nitrobacteriaceae (Buchanan, 1917), collectively known as nitrifiers, which undertake the nitrification reactions, include two discrete microbial partners tied faithfully to a life of biochemical harmony, namely ammonia oxidizers (nitritifiers) and nitrite oxidizers (nitratifiers). Each partner critically depends on the other. The two steps in nitrification can be summarized as follows:

$$NH_4^+ + 1.5\ O_2 \longrightarrow NO_2^- + H_2O + 2H^+ \quad G = -65\ kCal\ mol^{-1}\ N$$

$$NO_2^- + 0.5\ O_2 \longrightarrow NO_3^- \quad G = -18\ kCal\ mol^{-1}\ N$$

The overall nitrification reaction is as follows:

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H + H_2O$$

Schloesing and Muntz (1877) were the first to demonstrate the biological nature of nitrification by preventing nitrification in soil percolation columns by the addition of chloroform.

In 1890, Frankland and Frankland published a paper describing the isolation of a pure culture of an ammonia oxidizing, nitrite producing bacteria, in inorganic media by a process of terminal dilution. Winogradsky (1890) isolated a pure culture of nitrifying bacterium by 'inverse gelatine method'. Mineral based enrichment media was prepared whose pH was stabilized using sediment

magnesium carbonate, in which ammonia oxidizers grew in zoogloeas, which entrapped magnesium carbonate and form recognizable clots in the sediment. Some clots were washed with sterile water and placed on gelatin plates. Those containing heterotrophic contaminants produced colonies on the plates and autotrophic nitrifiers did not which produced tiny or no colonies in gelatin. He described the application of Von Kuhne silica gel medium (Von Kuhne, 1890), to nitrification. Winogradsky initiated isolation studies of nitrifiers on a series of soils from all over the world. (Winogradsky, 1892) His daughter Helene also joined hands and described more genera of nitrifying bacteria. Marine and terrestrial nitrifiers have been later described by other workers like Watson (1971 a, 1974). Soriano and Walker (1968), Belser and Schmidt (1978), Mac Donald (1979) , Walker and Wickramasinghe (1979), etc. The current taxonomy is based on the classical work of Sergei and Helene Winogradsky from 1890 to1937 and the more recent investigations by Watson and co workers from 1965 to1986.

The classification of the nitrifying bacteria is primarily based on morphological characteristics. Recent studies on the phylogeny of the nitrifying bacteria indicate that the family Nitrobacteriaceae include atleast three different groups of organisms which are not closely related to phylogenetically (Woese *et al.*, 1984 a, 1984 b, 1985). The nitrite oxidizing species of the ammonia oxidizing cells are usually Gram negative, ellipsoidal, or short rods and nitrite oxidizers are short wedge or pear shaped cells with flagellum and are Gram negative. The presence of cytochromes result in yellow or red suspensions. Both are obligate chemolithotrophs, with the exception of *Nitrobacter* which can grow mixotrophically. Additional species of nitrifying bacteria probably exist that have not been either cultured, isolated or described, since relatively few investigators have isolated nitrifying bacteria.

1.1.1 Cell biology of nitrifying bacteria

1.1.1.a Ammonia oxidizing bacteria

All species of these groups are Gram negative organisms, which are obligatory lithotrophic using ammonia as the sole energy source. They grow autotrophically with CO_2 as the main carbon source, but can also grow mixotrophically, assimilating organic compounds. Most species grow optimally at 25-30^{0}C, at pH 7.5-8 and ammonia concentration of 2-10 mM. The generation time varies from 8 hours to several days.

The cells of the species of *Nitrosomonas* are ellipsoidal or rod shaped with rounded or pointed ends. Most stains are motile with polar flagella and intracytoplasmic memebranes are arranged as flat ended vescicles in the peripheral regions of the cytoplasm. All strains of marine and brackish water genus *Nitrobacter* were members of α subdivision of the purple bacteria, the ammonia oxidizing species of *Nitrosomonas europaea*, *Nitrosococcus mobilis*, *Nitrosospira briensis*, *Nitrosolobus multiformis* and *Nitrosovibrio tenius* were in β subdivision of purple bacteria and *Nitrosococcus oceanus* in the γ subdivision (Woese *et al.*, 1985). It has been suggested that the genetic make up of their sub sets do not show evidence of a common genetic structure between ammonia oxidizers and nitrite oxidizers and each might have evolved from a distinctly different ancestral linkage.

The marine species are obligately halophilic. The marine strains have an additional cell wall layer outside the typical Gram negative cell envelope (Watson and Remsen 1969), strains of some species causes carboxysomes (Wullenweber *et al.*, 1977) while strains of other species do not possess polyhedral inclusion bodies.

Genus *Nitrosococcus* includes atleast three species *N. nitrosus*, *N. oceanus* and *N. mobilis*. *N. nitrosus* cells were spherical with a diameter of 1.5-1.7 μm. and non motile while *N. oceanus* were spherical to ellipsoidal, with diameter of 1.8 to 2.2 μm. and occur singly or in pairs with cysts observed in mixed cultures (Watson, 1965), and can be distinguished by centrally located stack of parallel, flattened membrane vesicles Watson and Remsen (1969), observed two additional cell wall layers outside the Gram negative cell envelope. Cells were generally motile possessing 1-20 polar flagella. *N. mobilis* cells were variable in size (1.2-1.9 μm. in diameter) when grown in diluted (2:3) natural seawater, occur singly, in pairs or as short chains. Intracytoplasmic membranes are present with an additional cell wall layer and cells are motile with a tuft of 1-22 flagella. All strains isolated so far are from brackish water and are obligately halophilic.

The genus *Nitrosospira* includes at least five species whose cells are tightly wound spirals with 3-20 turns and when motile, possess peritrichous flagella. Intracytoplasmic membranes are not observed and are distributed in soils and fresh water and not in marine habitats.

Genus *Nitrosolobus* has two species isolated from soil and from sewage. *N. multiformis* are pleomorphic motile with 1-20 peritrichous flagella, lobate in appearance , divided by constriction. Cells are partially compartmentalized by invaginations of the plasma membrane and other segments of cell envelope with 1-4 central compartments surrounded by 5-20 peripheral compartments. The second species isolated from sewage are smaller, 1-1.5 μm wide and 1-2.5 μm long.

Cells of *Nitrosovibrio* are slender, curved rods 0.3-0.4 μm by 1.1-3.0 μm., motile by 1-4 subpolar to lateral flagella and lack an extensive intra cytoplasmic membrane system. All strains were isolated from soil and a strain from an acidic

tea soil with pH of 4-4.5 was isolated by Walker and Wickramasinghe (1979). A second species has also been detected by DNA-DNA hybridization techniques.

1.1.1.b Nitrite oxidizing bacteria

These Gram negative organisms grow lithoautotrophically with nitrite as energy source and CO_2 as the main carbon source. Species of the genus *Nitrobacter* are able to grow heterotrophically on simple carbon compounds like pyruvate, acetate and glycerol. Most species grow optimally at 25-30^{0}C, at pH 7.5-8 and at nitrite concentrations from 2-30 mM. Generation time varies from 10 hours to several days.

Genus *Nitrobacter* consist of two species, *N. winogradskyi* and *N. hamburgensis*. *N. winogradskyi* are pleomorphic with a size of 0.6-0.8 x 1.2-2 μm. and motile with a single polar or lateral flagellum. The outer cell membrane is asymmetric the inner layer being more electron dense than the outer layer and peptidoglycan layer is not detectable. Intracytoplasmic membranes are arranged as a polar cap composed of 4-6 layers of paired membranes. The cytoplasmic side is stacked with 7-9 nm particles, which are reduced in number in resting cells and absent in heterotrophically grown cells. Cytoplasmic inclusions are carboxysomes, poly-β-hydroxybutyrate, glycogen and polyphosphates. *N. hamburgensis* are similar in shape, size and ultrastructure to *N. winogradskyi* and contain three different plasmids 117, 191 and 281 kb (Kraft and Bock, 1984). A third species with biphasic mixotrophic growth where first nitrite is oxidized, then, after a lag phase, organic substrates are metabolized is also there in the genus *Nitrobacter*.

Genus *Nitrobacter* consists of a single species, *N. mobilis* isolated from marine environment, which are 1.5-1.8 μm. in diameter and are generally motile with one or two flagella.

Clumps of hundred or more cells embedded in slime matrix have been observed. Intracytoplasmic membranes arranged as tubular invaginations of cytoplasmic membranes distributed randomly throughout the cytoplasm, poly-β-hydroxybutyrate granules and electron dense bodies believed to be glycogen storage products are observed.

Genus *Nitrospira* has one species isolated from marine environment, *N. gracilis* whose cells are long, slender rods of 0.3-0.4 x 1.7-6.0 μm. which in older cultures has spherical form of 1.35-1.45 μm. in diameter. Cells lack extensive intracytoplasmic membrane systems and when stained with glycogen specific stains, darkly stained bodies of 30-40 nm in diameter are seen.

In the genus *Nitrospira*, only species described, *N. marina* was isolated from marine environment is obligately halophilic and grows best mixotrophically on nitrite and pyruvate or glycerol. They are curved rods, which occur as tightly to loosely wound spirals with upto 20 turns when grown lithoautotrophically and with one turn when grown mixotrophically. Cell width is 0.3-0.4 μm. and amplitude of spirals is 0.8-1.0 μm. Cyst formation is frequently observed in enrichment cultures. Another strain has been cultured from soil sample from Namibia.

1.1.1.c Growth of nitrifiers

Growth of nitrifying bacteria is slow even under optimal conditions. Lithoautotrophic growth is maximum at 28-30^{0}C, at pH 7.6-7.8 and at a specific partial pressure of oxygen. Generation time vary from 8 hour for *Nitrosomonas* and 10 hour for *Nitrobacter* to 60 hour for *Nitrosospira.* The oxidation products, nitrite and nitrate are inhibitory for ammonia oxidizers and nitrite oxidizers respectively, the concentration of inhibition being different in different species. In lithoautotrophic cells of *Nitrobacter,* only 2-11% of the energy generated by

nitrite oxidation is used for the cell growth. Due to the low cell concentrations, estimation of biomass by absorbance technique is not normally possible.

Cell number increases in the presence of organic material and cultural filtrates of heterotrophic bacteria stimulate nitrite oxidation and increase cell yield (Steinmuller and Bock, 1976), and nitrification is often faster than in the pure cultures. Mixotrophic growth of different species of *Nitrobacter* exhibits different patterns. *N.hamburgenesis* uses nitrite and organic material simultaneously and the cell yield is high. Growth of *N.winogradskyi* is similar but less efficient while *Nitrobacter* sp shows diphasic growth, where nitrite and then organic materials are oxidised. *Nitrobacter* is also capable of anaerobic growth, reducing nitrate to nitrite with pyruvate, acetate or glycerol as electron donors. Visible, blue and long wavelength ,UV light are lethal to nitrifying organisms. (Muller- Neugluck and Engel, 1961; Schon and Engel, 1962). *Nitrobacter* is more sensitive than *Nitrosomonas* (Boch, 1965; Olson, 1981). Photoinhibition is due to photo oxidation of cytochrome C (Bock, 1965).

1.1.2 Biochemistry of nitrification

Ammonia oxidizing bacteria are obligate chemolithotrophs, oxidizing ammonia to nitrite, fixing carbon dioxide to fulfil their carbon and energy needs. The first step, the oxidation of ammonia to hydroxylamine is catalysed by a hydroxylamine to nitrite is catalysed by the hydroxylamine oxidoreductase. Carbondioxide is fixed via the Calvin cycle and serves as the main carbon source. 18 mol ATP and 12 mol reduced NAD(P) are required to fix 6 mol carbon dioxide. Although ammonia-oxidizing bacteria can grow mixotrophically on several organic compounds, (Krummel and Harms, 1982), heterotrophic growth has never been observed.

Nitrobacter species, when grown lithoautotrophically oxidize nitrite to nitrate thus producing ATP and NADH for CO_2 fixation via the Calvin cycle. The source of oxygen for nitrite oxidation has been shown to be water (Aleem *et al.,* 1965). The nitrite oxidizing system is membrane bound and the membranes possess a brownish colour, which is typical for all nitrite oxidizers. The enzyme responsible for nitrite oxidation is nitrite oxidoreductase.

1.1.3 Inhibition of nitrification

Hauck (1980), listed 17 pesticides known to inhibit nitrification and 13 chemicals that have been developed commercially as specific inhibitors of nitrification for inhibiting nitrification in soils to minimize losses of nitrate by leaching or denitrification . Nitrapyrin, allylthiourea, diethydithiocarbamate acetylene and ethyl xanthanate are specific inhibitors of nitrification which inhibit the cytochrome oxidase in turn inhibits the nitrification reaction. There are also non-specific inhibitors of nitrification, which are chemicals that affect the growth and proliferation of bacteria. Nitrifiers are notoriously sensitive to S-containing aminoacids. Carbon disulfide and other reduced sulfur compounds, thiocarbamates and thiosulfates are inhibitory. Ammonium oxidizers are partially affected by dithiocarbamates that release CS_2. Some plant products like karanjin, a flavonoid from the karanjin seed (Sahrawat, 1981), and Margosa seed cake (Mishra *et al.*, 1975), also inhibit nitrification.

1.1.4 Heterotrophic nitrification

In addition to autotrophic nitrification many heterotrophic microorganisms can convert both organic and inorganic reduced nitrogen compounds to nitrate. But chemoautotrophic process is the dominant one. Heterotrophic nitrifiers include fungi (Odu and Adeoye,1970), heterotrophic bacteria (Nelson, 1929; Cutler and Mukerji, 1931) and actinomycetes (Remacle, 1974). Among fungi, *Aspergillus flavus* shows significant nitrification. Numerous fungi isolated from

acid coniferous forest soils have the ability to nitrify like a species of *Penicillium* isolated from Germany (Remacle, 1977). Nitrification products appear to accumulate in heterotrophic nitrification when active growth ceases (Schmidt, 1960) and the energy gain by the process is very low. Certain of the heterotrophic nitrification products are known to be toxins and mutagens. A key role of heterotrophic nitrification lie in the production of compounds such as hydroxamic factors (Verstraete, 1975) and have been implicated in the uptake of iron by microorganisms.

1.1.5 Adhesion and biofilm formation of nitrifying bacteria

The adhesive properties of nitrifying bacteria have traditionally found application in biological filters. Ardakani *et al.*, (1974) has observed that nitrifying bacteria are located to a large extent at the surface of soil and suspended particles. Underhill and Prosser (1987), have shown that ion exchange resins or glass surface enhanced the specific growth of *Nitrosomonas europaea* and *Nitrobacter* sp.cells and attributed this to attachment of bacteria on surfaces. Diab and Shilo (1988), found enhancement in nitrifying activity as a result of attachment *Nitrosomonas* and *Nitrobacter* cells incubated under aerobic conditions in the absence of ammonia or nitrite was considerably longer than that of free cells. Physiological processes upon attachment changes based on nutrient concentration and/ or nutrient availability at the attachment site, modification of cell membrane associated process and formation of a polymeric matrix, which may affect the interactions between cells and protect against access of toxic substances, phages and even predators.

Kholdebarin and Oertli (1977), concluded in their study that nitrification is enhanced in the presence of particles which increases the area for physical attachment of bacteria. Nitrifiers are found to embed their cells within zoogloea and most of the bacteriological activity in filters occurs inside living films attached to solid surfaces. Thus they gets attached to the substratum and act upon

the nutrients which come into contact with the cells. Alleman and Preston (1991) has opined that by getting attached, nitrifiers may be conserving their energy.

1.2 Major nitrogen removal processes

Major processes for nitrogen removal are air stripping (ammonia stripping), breakpoint chlorination (superchlorination) selective ion exchange and biological nitrification

1.2.1 Air stripping

Ammonia is a gas, which dissolves in water, and the dissolution controlled by the partial pressure of ammonia in the air adjacent to water. The molecular state of ammonia is highly pH dependent and in alkaline pH, it exists in the unionized form (NH_3). In alkaline pH (10-11) when partial pressure is reduced ammonia leaves the liquid phase and enters the gaseous phase. Ammonia can be stripped from wastewater by bringing small drops of water in contact with a large amount of ammonia free air. This process does not affect nitrite, nitrate and organic nitrogen. The disadvantages are its inefficiency in cold weather and formation of calcium carbonate scales in air stripping towers.

1.2.2 Breakpoint Chlorination

This is a highly efficient method of ammonia removal with absolute removal efficiencies. Chlorine is added to the wastewater in an amount to oxidize ammonia to nitrogen gas. After sufficient chlorine is added to oxidize the organic matter and other oxidizable substances, a stepwise reaction of chlorine with NH_4^+ takes place as follows:

$$3\,Cl_2 + 2\,NH_4^+ \longrightarrow 6\,HCl + 2\,H^+ + N_2$$

In practice, approximately, 10 mg L^{-1} of chlorine is required for every 1 mg L^{-1} of ammonia-nitrogen. The acidity produced has to be neutralized by the addition of caustic soda or lime which adds to the total dissolved solids and increases the operating expense. Also the health effects of breakpoint chlorination or superchlorination products are highly controversial and this process does not remove nitrate.

1.2.3 Selective ion exchange

Ammonia removal of 90-97% is accomplished by this method. Here the wastewater is passed through a column of clinoptilolite, a naturally occurring zeolite, which has a high selectivity for ammonium ion at a pH of 6.5. When all the exchange sites are utilized, regeneration can be done. Nitrite and nitrate cannot be removed by this process.

1.2.4 Biological nitrification

Removal of ammonia in biological nitrification is by transformation of ammonia to nitrate via nitrite undertaken by bacterial cells. It can be carried out in conjunction with secondary or tertiary treatment using either suspended growth reactors (activated sludge) or attached growth reactors (trickling filters etc)

1.2.4.a Activated sludge process

In activated sludge process, wastewater is brought into contact with a mixed microbial population in the form of a flocculant suspension with sufficient aeration. Suspended and colloidal matters are removed rapidly by adsorption and agglomeration onto the microbial flocs. This and the nutrients are broken down slowly by microbial metabolism and oxidized to simpler products such as carbon dioxide. Endogenous respiration also plays a part. Then the sludge is removed in clarifier or settling tanks. A part of sludge is reused as microbial inoculum and

the rest is disposed off. This is the most widely used process for treatment of organic and industrial wastewater and a number of modification on the basic process has been done to make it move versatile.

1.2.4.b Trickling filter

The wastewater is allowed to trickle or percolate through a stationary bed of stones, gravels etc., which serve as an attachment surface for microbes. The water comes through a nozzle, the sprinkle rotates and the water is sprinkled all over the bed. The surface in contact with the nutrient rich wastewater containing microbes will develop a biologically active slime layer. The dissolved oxygen is transferred to the microbial film and the oxygen and nutrients from the water diffuse into the microbes in slime. Trickling filters are stable, simple and fairly fool proof in design.

1.3 Application of nitrification in hatcheries

1.3.1 Ammonia and nitrite formation in hatcheries

Nitrogen, as ammonia enters hatchery seawater through diffusion from the atmosphere and subsurface air bubbles, excretion by prawns, oxidation process of heterotrophic bacteria and lysis of bacterial cells. Nitrite occurs due to ammonia oxidation by ammonia oxidizers.

The balance between unionized and ionized ammonia is highly pH dependent. As pH increases by one unit, the amount of toxic unionized ammonia increases about 10 times. A decrease in dissolved oxygen increases the toxicity of un-ionized ammonia.

1.3.2 Factors affecting nitrification

1.3.2.a Oxygen tension

Every milligram of nitrogen passed through their full nitrification pathway from ammonia to nitrite requires approximately 4.5 mg of dissolved oxygen (Alleman and Preston, 1991), to scavenge electrons drawn from their nitrogenous substrates. If dissolved oxygen drops below a few mg per litre, nitrifier metabolism will markedly slow down.

1.3.2.b pH and alkalinity

Nitrifying bacteria prefer an alkaline pH range between 7 and 8. Alkalinity levels adequate to stop pH from dropping below the preferred alkaline range is required for nitrification to proceed smoothly.

1.3.2.c Temperature

Nitrifiers prefer moderate to warm temperatures, ranging from 28-38^{0}C. The growth constants of nitrifying bacteria were affected greatly by temperature (Sharma and Ahlert,1977)

1.3.2.d Salinity

Nitrifiers have a sizeable range of tolerable osmotic pressures, ranging from fresh to saline, depending on the particular genus form. Many nitrifiers are able to rapidly switch from one salt level to another with little impact on their activity.

1.3.2.e Light

Nitrifiers are sensitive to visible, blue and long wavelength U.V. light. Photoinhibition has been demonstrated to be due to photooxidation of cytochrome C (Bock, 1965).

1.3.3 Ammonia and nitrite toxicity and management practices in hatcheries

In general, the concentration of ammonia (as total NH_4^+) should not exceed 0.1 ppm. Sublethal toxicity results in reduction in growth rate. Exposure to ammonia causes gill hyperplasia, which is a precursor to bacterial gill diseases, and in extreme cases, causes death. Concentration of nitrite should not be greater than 1 ppm in prawn rearing ponds. Nitrite toxicity causes brown blood disease, growth retardation and death.

More and more hatchery operators are now-a-days preferring closed system hatchery, management to avoid the risks of disease outbreaks and predator invasions, so the management of toxic metabolites like ammonia is a major problem faced by aquaculturists. Daily specific excretion of ammonia by post-larvae is about five folds higher than excretion by adults. In hatcheries, there should be a proper technology for removal of ammonia, which could other wise lead to a catastrophe.

The usual practice in hatcheries to remove ammonia and nitrite is by using bacteriological filters. In a bacteriological filter, solid particulate media is held in a container over which water circulates. The nitrifying bacteria attached to the media acts upon ammonia or nitrite and oxidizes them to NO_3. But the main drawback with filters is the long conditioning period required for establishing good nitrifying potential or spiking the water with nutrients. Inoculates of nitrifying bacteria for addition to newly established filter beds can be obtained from pure laboratory cultures, commercial suppliers, in a freeze-dried state or

from conditioned filter beds of similar temperature and salinity. Nitrifying bacteria are difficult to be cultured. Also, laboratory cultures require time to adapt to the hatchery conditions. With freeze dried products, the results have proved inconsistent (Spotte, 1992). The easiest way now available is to add a handful of old gravel from a conditioned filter bed. But this works only when again, the chances of transferring disease causing organism along with the sand is there. When a biological filter is attached to a hatchery system it becomes difficult to subject it to disinfection programmes as the nitrifiers will be totally washed out and it would take yet another couple of months for the conditioning of the filter. More over the conventional biological filters entrap larvae and feed particles and make the whole process non-viable. Therefore the need for a viable user friendly and commercially viable technology for the rapid and continuous removal of ammonia and nitrite from penaeid and non-penaeid hatchery system has been felt for very long time and the present research programme was conceptualized and executed that end. The work was oriented towards developing bioreactors for nitrifying the larval rearing water continuous by, during larval rearing itself and also for nitrifying freshly collected sea water and spent water as that the water can be reused and there by system can be made a closed and recirculating one.

As part of maintenance of biological filter detritus has to be removed from the filter bed every two weeks after stirring the media, usually gravel. Some of the filter bacteria may be removed along with excess detritus; then the population density decreases and sometimes a detectable lag in nutrient conversion may occur.

Hence, this work is an attempt in this direction to cater to the needs of aquaculture industry for treatment and remediation of ammonia and nitrite in penaeid and non-penaeid hatcheries, by developing nitrifying bacteria allochthonous to the particular environment under consideration, and immobilizing them on an appropriately designed support materials configured as reactors.

CHAPTER 2

DEVELOPMENT OF NITRIFYING CONSORTIA FOR THE NITRIFYING BIOREACTORS

CHAPTER 2

DEVELOPMENT OF NITRIFYING CONSORTIA FOR THE NITRIFYING BIOREACTORS

2.1 Introduction

Exhaustive literature is available on the isolation of nitrifying bacteria from marine and brackish water environments using different kinds of media of varying composition. For marine ammonia oxidizing bacteria, Soriano and Walker (1968), Carlucci and Strickland (1968), Watson (1965), Watson and Mandel (1971), Hooper and Terry (1974), Goreau *et al.*, (1980) and Belser and Mays (1982) suggested various media and for estuarine ammonia oxidizers, Helder and DeVries (1983) suggested specific media and several others on the other hand used Winogradsky media described by Rodina (1972) with amendment of salinity and with the addition of appropriate volumes of artificial seawater. Same media were used for estuarine nitrite oxidizers also with only substrate change. Watson and Waterbury (1971) and Watson and Mandel (1971) suggested two different enrichment media for marine nitrite oxidizing media. Most of the media suggested contained in addition to the substrates in the form of NH_4Cl or $(NH_4)_2SO_4$ for ammonia oxidizers and $NaNO_2$ for nitrite oxidizers magnesium, calcium and phosphate salts with chelated iron and other metals like cobalt, copper, zinc, manganese and molybdenum in trace quantities. Meanwhile certain simple media were also described by Watson, 1965. pH of all media ranged between 7 and 8.

Biofilters have been traditionally used in aquaculture for removal of ammonia where attachment sites are provided by gravel. When a new filter is set up, it takes almost 30 to 60 days for nitrification to set in. Therefore it is a

traditional knowledge that nitrifiers get attached to substratum and gradually the cell number increases and nitrification sets in. Thus an active biofilter can serve as a rich source of nitrifiers.

It has been observed by Kawai *et al.*, (1964) that nitrifiers in the filter bed of a conditioned biological filter can support hundred times more nitrifiers than that are available suspended in water. The sand grains of a conditioned biofilter could then be a rich source of chemolithotrophic nitrifiers (Ramachandran and Singh, 1996 and Ramachandran, 1998). If the initial number of nitrifiers in the source material happens to be small, there is every possibility of loss of organisms while sampling. In such instances, it is advisable to develop pre-enrichment systems from which nitrifiers can be easily isolated. Biological filters can function as an effective pre-enrichment system for nitrifiers, when they are present in low numbers in water column. This principle was employed in the present work for the enrichment and isolation of nitrifiers.

An airlift pump is the most trouble free means of moving water through a biological filter. An airlift is essentially a central pipe through which air water mixture is moved upward. In culture applications, part of the pipe extends below the subgravel filter plate, a portion of it extends above the filter plate, and the air is injected at the lower end of the pipe. As long as air is injected, the air-water mixture is spilled at the top of the pipe (Spotte, 1979)

A culture system is considered to be conditioned when the nitrifying bacteria in its biological filter can rapidly oxidize all the incoming ammonia to nitrate with no significant appearance of the intermediate nitrite (Bower and Turner, 1983). These sandgrains of the filter bed of a conditioned biofilter can be used as a rich source of chemolithotrophic nitrifiers for further enrichment and isolation.

Lewis and Pramer (1958) were the first to use particle free media for the isolation of *Nitrosomonas* species. They prepared enrichment cultures either by successive transfers of fully grown cultures to fresh medium or by the addition of more ammonium ion after the oxidation of the initial amount present was exhausted using the latter method, they found an initial increase in heterotrophs, a decrease in numbers following the first addition of fresh ammonium and relatively constant numbers following the second addition. Ammonia oxidizers were found to outnumber heterotrophs by a factor of 50.

Although no organic compounds are added to the enrichment media, the nitrifying bacteria excrete organic compounds that can support the growth of heterotrophic bacteria. Moreover nitrifiers are slow growers. Therefore isolation of nitrifying bacteria is a difficult and time-consuming task. Even if pure cultures of nitrifiers are used for developing bioreactors, which are to be used in outdoor aquaculture conditions, they will be contaminated by heterotrophs. So an integrated approach on culturing nitrifying bacteria and associated heterotrophs as a mixed culture consortia or nitrifying consortia is followed in this study.

Several media have been proposed by earlier workers for enrichment of nitrifiers. Most of them contain, in addition to $(NH_4)_2SO_4$ and $NaNO_2$ as substrates, varying quantities of Magnesium, Calcium, phosphates and trace metals. According to the salinity requirements they suggested distilled water or seawater or a combination of both.

Belser and Schmidt (1978), evaluated several media and reported the media of Soriano and Walker (1968), to be superior to others tested, for ammonia oxidizing bacteria from soil. Our primary consideration was a simple enrichment medium capable of supporting efficient growth of nitrifiers. An initial enrichment medium suggested by Watson (1965), consisted of NH_3-N and PO_4-P. Another medium by the same author had in addition $MgSO_4.7H_2O$, $CaCl_2$ and trace

metals. An enrichment medium suggested by Carlucci and Strickland (1968) was also found to be very simple.

Suzuki *et al.,* (1974) suggested that ammonia oxidizer used unionized ammonia, NH_3, in a gaseous state. But most textbooks indicate that nitrification starts from ionized ammonium-nitrogen, NH_4^+-N. In this study NH_4^+-N is considered to be the starting point of nitrification.

Enrichment cultures can be obtained from soils, compost piles, sewage, fresh water, salt water, aquatic sediments or other appropriate sources. Watson *et al.,* (1981) suggested that where a 1% inoculum was used, a lag phase of a few days to few weeks were observed. The activity of nitrifiers can be detected by monitoring the substrates and the products. The oxidation of ammonia can be detected by an increase in the nitrite or nitrate concentration in culture and oxidation of nitrite, by detection of nitrate. A lowering of pH is also indicative of nitrification (Watson *et al.*, 1981).

In enrichment cultures, nitrifiers occur in cell aggregates, referred to as zoogloea or cysts. A zoogloea contains loosely associated cells embedded in a soft slime layer, while a cyst contains closely packed and compressed cells embedded in and surrounded by a tough slime layer. Those aggregates may consist of a few to a hundred or more cells. Both ammonium oxidizers and nitrite oxidizers are Gram negative organisms.

By comparison with heterotrophs the growth of nitrifiers is a slow process. Generation time of the ammonia oxidizing bacteria reported 8 hours to several days even under optimal conditions and for nitrite oxidizers from 10 hours to several days. Generation times vary from 8 hour for *Nitrosomonas* and 10 hours for Nitrobacter to 60 hours for *Nitrosospira*. The oxidation products, nitrite and nitrate are inhibitory for ammonia oxidizers and nitrite oxidizers, respectively. The minimal concentration for inhibition varies between species (Bock *et al.*,

1986). At maximal activity levels, nitrifying bacteria consume several times body weight in substrates each day, but, convert far less of their consumed substrate into new cell mass. Thus cellular yield is considerably low for nitrifiers (Alleman and Preston, 1991).

Nitrosomonas and *Nitrobacter* oxidize about 35 and 100 atoms of nitrogen for fixation of a molecule of CO_2 (Alexander and Clark, 1965). About three times as much nitrogenous substrate is thus required for the growth of nitrite oxidizing bacteria than for ammonia oxidizing bacteria because the resulting free energy change (Δ F) is about –65 Vs. –20 KCal/mol for the oxidation of ammonium and nitrite, respectively. Despite the fact that larger growth yields are evident from a mole of ammonium than a mole of nitrite, the numbers of ammonium oxidizing bacteria do not always appear to be larger than nitrite-oxidizing bacteria in nature. Morrill and Dawson (1967), observed that the number of *Nitrosomonas* were slightly larger than *Nitrobacter*, though this varied depending on the pH of the soil. The relationship of nitrite oxidation to cell density appears to be valid only for growing cultures since oxidation decreased as the cells become older (Hofman and Lees, 1952; Gould and Lees, 1960). Maximal nitrite oxidation activity has been reported to decline 40 hours before the maximal cell density is reached with both *N. agilis* and *N. winogradskyi* (Fliermans and Schmidt, 1975)

In nature, growth rate of nitrifiers is controlled by light, substrate (ammonia and nitrite), temperature, pH and oxygen tension. Most strains of nitrifying bacteria grow optimally at substrate concentration of 1-25 mM, pH between 7.5 and 8 and a temperature of 25-30^{0}C. However, many environments with suboptimal conditions still support the growth of nitrifying bacteria. For example, nitrifying bacteria are strict aerobes, yet they can be isolated from sewage-disposal aeration tanks that are extremely low in oxygen. They can also be isolated from soils with a pH of 4, but cannot grow at pH less than 6.

Visible, blue and long wavelength and U.V. light are lethal to nitrifying organisms (Muller-Neugluck and Engel, 1961) ; *Nitrobacter* is much more sensitive than *Nitrosomonas* (Bock, 1965; Olson, 1981). Light partially inhibited the growth of *N. oceanus* (Watson, 1965). He found that in shake culture flasks exposed to normal laboratory light, generation time is 84 hours while for flasks incubated in dark it was 22 hours. Inhibition is caused by wavelengths below 480 nm (Schon and Engel, 1962) and the mechanism of light inhibition is apparently photo-oxidation of cytochrome C (Bock,1965). Recovery from light inhibition required several days. Yoshida and Saijo (1985) reported that 'several tens of days' in the dark were necessary for recovery from light inhibition for ammonium and nitrite oxidizers isolated from lake waters.

Nitrifiers need oxygen for their normal metabolism. Every milligram of N_2 passed through their full nitrification pathway (starting at ammonia and concluding at nitrate) requires approximately 4.5 mg of dissolved oxygen to scavenge electrons drawn from their nitrogenous substrates. At low oxygen tension, nitrifier metabolism will markedly slow down. However, even without access to any O_2, they can sustain some measure of respiratory metabolism using nitrate or nitrite as an alternative electron acceptor (Alleman and Preston, 1991). Reduced rates of nitrification in sewage occurred at O_2 levels of 1 mg L^{-1} and the concentration remained steady above 2 mg L^{-1}. Studies of axenic culture stress the importance of adequate aeration for active nitrification. Aeration of cultures of nitrifying organisms is important not only to provide oxygen but also for the supply of carbon dioxide, unless carbonate is used to neutralize acid production. Saturation constants for oxygen uptake are in the range of 0.3 to 1.0 mg O_2 L^{-1} with *Nitrobacter* sp. slightly more sensitive to oxygen limitation than *Nitrosomonas* sp. (Prosser and Cox, 1982)

Growth and metabolism of the autotrophic nitrifying bacteria are optimal in neutral to slightly alkaline pH range (pH 7 to 8). The pH range for complete nitrification is very restricted due to toxicity of free ammonia at alkaline pH and nitrous acid at acid pH respectively (Anthonisen *et al.*, 1976). Growth of

ammonia oxidizers is autoinhibitory since 2 mol H^+ ions are produced for each mol ammonia oxidized. Inhibition of *Nitrobacter* sp. at low pH is due to nitrous acid, while high pH results in dissociation of nitrite from an active site of the enzyme complex as a result of competition by hydroxyl ions (Boon and Laudelout, 1962). The concentration of free ammonia or undissociated nitrous acid is pH dependent. The pKa for NH_4/NH^+ is 9.3, and the pKa for HNO_2/NO_2^- is 3.4 .

The favourable range of both *Nitrosomonas sp.* and *Nitrobacter sp.* is pH 7 to 9 with inhibition below pH 6, although slow rates of nitrification have been reported at pH values as low as 4.5. But as earlier said, those cultures isolated at pH 4 could not grow at a pH less than 6. Nitrobacter will grow in pure culture upto a pH of 10.2 (Meyerhof, 1917) when NO_2^- is the only nitrogen source present. Prakasam and Loehr (1972) found that nitrification was unaffected upto pH 11.2 as long as free ammonia concentration was less than 0.02 $\mu g\ mL^{-1}$.

Aside from the ambient pH itself, nitrifiers appear able to handle rather sizeable dynamic transients in this parameter. However, after acclimation, it would be best for their activity if the pH is kept as consistent as possible. Alkalinity levels adequate to stop pH from dropping below the preferred alkaline range was adequate for nitrifiers as noted by Alleman and Preston (1991).

The optimum temperature for nitrification in pure culture ranges from 25-35^{0}C (Meiklejohn, 1954) with no significant growth below 5^{0}C or above 40^{0}C, although Laudelout and Van Tichelen (1960) reported maximum growth of *Nitrobacter winogradskyi* at 42^{0}C and some growth upto 49^{0}C. The failure of autotrophic nitrifiers to grow above 40^{0}C is generally believed to explain why nitrate or nitrite is not found in thermophilic composts.

Temperatures above 35^{0}C are generally of minimal significance to most aquatic and terrestrial systems. Greater importance is there for the effect of lower temperature (0-15^{0}C), which is fairly common and difficult to assess quantitatively during cooler months of the year. Nitrification was more rapid

under field conditions at low average spring temperatures than in the laboratory, the explanation being that diurnal fluctuations in temperature affected greater amounts of available nitrogen. Soils subjected to fluctuating square-wave temperatures below 15.5^0C supported more nitrification than soils held at the constant mean temperature. Even when autotrophic nitrifying bacteria apparently become acclimated to the temperature regime of their habitat and do not appear to vary in their adaptability to low temperatures (Focht and Verstraete, 1977), their metabolic activity declines at lower temperatures and at higher temperatures they enter a region of potential life threatening stress, perhaps due to enzyme disruption (Alleman, 1991)

Nitrifiers have a sizeable range of tolerable osmotic pressure, ranging from fresh to saline, depending on the particular genus. Furthermore, many nitrifiers seem able to rapidly switch from one salt level to another with little impact on their activity (Alleman and Preston, 1991). Somville (1984) found that nitrifier population from fresh water display an optimum activity at a very low chlorinity and their activity rapidly decreases with increasing chlorinity. Nitrifier populations from brackish water samples have optimum activity at 7 g Cl^- per litre, i.e., at a salt content slightly lower than that in the water where they live. At sea water salinity, potential activity never falls below 50% of optimum. Finstein and Britzski (1972) have shown that the nitrifiers adapt to salinity during long term incubations, although different degrees in salt tolerance were noted among the selected populations.

Rates of ammonium and nitrite oxidation tend to be dependent on the substrate concentration because K_m is usually equal to or larger than substrate concentration in nature. K_m for ammonia oxidation ranges between 1 and 10 mg N L^{-1} between $20\text{-}30^0$C (Hofman and Lees, 1952; Loveless and Painter, 1968) while K_m for nitrite oxidation falls between 5 and 9 mg L^{-1} in the same temperature range.

Substrate inhibition of the nitrifying bacteria has been observed at relatively high levels (Boon and Laudelout, 1962; Watson, 1971; Prakasam and Loehr, 1972) and is pH dependent since the inhibition is effected by free ammonia or undissociated nitrous acid. End product inhibition by nitrite to *Nitrosomonas* is an apparently more complex mechanism since lower concentrations (500 mg N L^{-1}) are more toxic during the lag phase (2500 mg N L^{-1}) (Painter, 1970). Nitrite concentrations of 1400 and 4700 μg N mL^{-1} effect a 26 and 100 % inhibition of oxygen uptake by washed cell suspensions of *Nitrosomonas.* Watson *et al.,* (1981) observed inhibition of nitrite to *Nitrosolobus* at 1400 μg N mL^{-1}. High concentrations of nitrate appear to non competitively inhibit the oxidation of nitrite by *Nitrobacter* (Boon and Laudelout, 1962). Gould and Lees (1960) found that greater cell densities could be maintained by continuous removal of nitrate by dialysis or by continuous culture. The intimate association of nitrification with carbon dioxide fixation may account for the greater rates of ammonium or nitrite oxidation and the lesser sensitivity to end product inhibition observed by growing cells. Resting cells of *Nitrobacter*, for example, lose the ability to oxidize nitrite, but by supplying nitrite, oxygen and carbon dioxide, the oxidative activity returns. Similarly the rate of nitrite oxidation is drastically reduced when the stationary growth phase is reached (Fliermans *et al.*, 1974; Fliermans and Schmidt, 1975).

Autotrophic nitrifiers depend entirely on the oxidation of nitrogen as their energy supply. It is highly unlikely that heterotrophs gain a significant amount of their overall metabolic energy from their nitrification process. Even if it happens, the net amounts of energy trapped from these nitrogen oxidation would be small compared with those of the normal organic carbon oxidation since these organisms oxidize only small amounts of nitrogen. However, the heterotrophs can obtain energy from the carbon moiety of certain nitrogenous compounds which they metabolize (Focht and Verstraete, 1977).

Stock cultures of nitrifiers are maintained at low temperature (15^0C) in appropriate medium with periodical analysis of substrate and addition of

increments when needed. Prior to subculturing, the cultures should be incubated at 25^0C for several days. (Watson *et al.*, 1981). Nitrifying bacteria have slow decay and death rates and they can be shifted into a dormant or resting state for extended periods with less concern about retaining their viability (Alleman and Preston, 1991).

Enumeration of and thereby assessment of nitrifying potential of nitrifiers was always difficult because normal plate counting methods could not be used since ammonia oxidizers do not form visible colonies within an acceptable period of time. Same is the case with nitrite oxidisers. A most common method used to enumerate viable nitrifiers is the most probable number technique (MPN) (Morrill and Dawson, 1967) and has been applied and improved by many (Matulevich *et al.*, 1975; Sarathchandra, 1979). But this requires incubation periods of about 11 weeks (Belser and Schmidt, 1978). Another method is the potential nitrification assay by Belser and Mays, (1982). In this the ammonia oxidizing enzyme activity is measured at optimal substrate concentration during a short term incubation (4-8 h). Many reports refer to discrepancies between the MPN count and potential enzyme activity in natural environments (Belser and Mays, 1982). Ramachandran (1998) described a similar method for calculating nitrifying potential of nitrifiers based on quantity of NO_2^-. N produced for ammonia oxidizers and quantity of NO_2^-. N consumed for nitrite oxidizers, giving all the optimum conditions required for their optimum growth within a short period. This is the method followed in this study for assessing the nitrifying potential.

The easiest method of seeding a culture system with nitrifiers is to inoculate with a pure culture or consortium from a culture collection centre. But it is feared that such introduction of nitrifiers isolated from dissimilar ecosystem and alien waters may not be a successful venture as the changed environmental parameters may decline the activity or else the organisms may undergo a prolonged lag phase. The only way to tide out this difficult situation is to develop and establish a consortium of nitrifiers from similar aquatic systems. In this context the consortia of nitrifiers developed from prawn grow-out systems as well

as from oceanic environment will find a ready application in prawn hatchery as well as in grow-out systems for achieving nitrification at desired levels (Achuthan and Singh, 1999).

To facilitate the development of bioreactors for nitrifying water in closed system prawn hatcheries it is essential to have suitable nitrifying consortia, mass culture them and make them available for further processes.

This chapter deals with the achievements made in

1. Pre-enrichment of nitrifiers in biological filters, enrichment in different media, selection of suitable media for the enrichment.
2. Selection of suitable consortia for penaeid and non-penaeid system.
3. Mass culture of nitrifiers in fermentor.
4. Determination of optimum pH, temperature, substrate concentration and salinity for the selected consortia.
5. Determination of nitrifying potential of these consortia, and
6. Maintenance of the nitrifying consortia.

2.2 Materials and Methods

2.2.1 Pre-enrichment in Biological filters

The source of nitrifiers was identified taking into consideration the differing salinity requirements in penaeid and non-penaeid hatcheries. Since in penaeid hatcheries salinity maintained or required is at around 30 ppt during the larval stage the water samples were collected from regions where around 30 ppt used to be perpetuating throughout the year. The samples and sampling stations were (1) fresh sea water from Kannamali, Kochi, (2) water from penaeid grow out

system of Shrimp Farm and Training Centre of Aquaculture Development Agency, Kerala, at Maala, Trichur, (3) Water from a prawn grow out system at Aayiramthengu, Alleppey, and, (4) Filterent grains of a biological filter set up in this laboratory for penaeid prawn maintenance. On the other hand, in non-penaeid hatcheries especially meant for *Macrobrachium rosenbergii* the normal salinity maintained is around 13-15 ppt. To match this requirement the source of the nitrifiers were fixed at regions where the salinity remains between 10-15 ppt throughout the year. Accordingly the samples and sampling stations were (1) Water sample from a prawn grow out system at Maradu, Cochin, (2) Filterent grains of a biological filter of a prawn larval rearing system at Shertallai, Alleppey, and, (3) Filterent grains of a biological filter of a prawn maintenance system set up in this laboratory. Water samples were collected and transported to the laboratory in polythene carboys under ambient temperature. Filterent grains from biological filters were collected in polythene bags and transported to the laboratory by maintaining in thermocool boxes.

All water samples were subjected for a pre-enrichment in a biological filter set up in the laboratory (Fig.2.1). This consisted of a 10L plastic basin to hold the water samples brought from various locations and a small basin of 1L capacity to serve as the biological filter. In this basin 1-cm thick coarse sand is layered and a plastic funnel is kept inverted, subsequent to which the space is filled with sand. An air sparger made in glass is placed inside the funnel and air passed through it, which served, as an air lift pump. The filter bed was kept submerged in the water sample. Air from an air compressor was passed through a sterilizable cotton filled pipeline filter prior to its passage through the airlift pump. The outer basins were filled with water samples brought from different locations and supplemented with $(NH_4)_2SO_4$ to attain a final concentration of 10 μg NH_4^+-N mL^{-1}. Every 24 hours, estimation of ammonia and nitrite following Bendschneider and Robinson (1952) and Solorzano (1969) and nitrate following the method of hydrazine sulphate reduction (Strickland and Parsons, 1968), modified to suit seawater were made with adjustment of pH to 7.5 using 10%

sodium carbonate. With the exhaustion of 90% NH_4^+-N fresh aliquots of $(NH_4)_2SO_4$ were added aseptically. After a conditioning period of 90-160 days, when the total quantity of NH_4^+-N oxidized was around 200-300 mg mL^{-1}, aliquots of sand grains of the filter bed were also used as the source of nitrifiers for subsequent enrichment. Meanwhile the samples of filterent grains obtained from other biological filters available elsewhere were used readily for subsequent enrichment.

2.2.2 Assessment of the adhesion of nitrifiers on sand grains of the filter bed

Sand grains from the conditioned biological filter were washed gently with sterile seawater of the same salinity and fixed in 3% v/v glutaraldehyde for 24 hours at 4^0C. After decanting the glutaraldehyde, sand grains were dehydrated using graded acetone (30%, 50%, 70%) giving 15 minutes each at every transfer. The fixed and dehydrated grains were kept at 4^0C in 70% acetone till observations were made. Air dried sand grains were placed in EM stubs and coated with gold for three minutes in a sputter coater. Then they were observed on Scanning Electron Microscope (H 600, Hitachi Ltd., Japan) and photographed.

2.2.3 Selection of appropriate media

For the isolation of nitrifying bacteria, several media were proposed by earlier workers. Based on simplicity in composition, three media were selected for enrichment of the nitrifiers.

Medium I (Simplified nitrification medium according to Carlucci and Strickland, 1968)

Water sample	:	100 mL
NH_4^+-N / NO_2^--N	:	10 mg
K_2HPO_4	:	2 mg
$NaHCO_3$	:	5 mg

pH : 8

Into the water sample, all the ingredients, which were sterilized by autoclaving, were added. Here water samples were directly used from the sites without going in for pre-enrichment in the biological filters.

Medium II (Watson, 1965)

Sea water	:	100 mL
NH_4^+-N / NO_2^--N	:	10 µg
PO_4-P	:	2 µg
pH	:	8

Medium was autoclaved at 15 lbs. for 15 minutes and inoculated with water/sand from filter bed.

Medium III (Watson, 1965)

Sea water	:	100 mL
$(NH_4)_2SO_4$ / $NaNO_2$	:	10 µg
$MgSO_4.7\ H_2O$	:	20 µg
$CaCl_2$	:	2 mg
K_2HPO	:	11.4 mg
Chelated iron	:	30 µg
$Na_2MoO_4.2H_2O$	:	0.1 µg
$MnCl_2.4H_2O$	:	0.2 µg
$CoCl_2.6H_2O$	:	0.2 µg
$CuSO_4.H_2O$	:	2 µg
$ZnSO_4.7H_2O$	:	10 µg

Medium was autoclaved at 15 lbs. for 15 minutes and inoculated with water/sand from filter bed.

All the three media were used for enrichment of ammonia oxidizers and nitrite oxidizers separately, only difference being the substrate, i.e., $(NH_4)_2SO_4$ was used in the case of ammonia oxidizers and $NaNO_2$ in the case of nitrite oxidizers.

Enrichment was done in 250-mL flasks, and the flasks were incubated on a rotary shaker at 100 rpm under obscurity.

2.2.4 Primary, Secondary and Tertiary enrichment

Water samples collected for enriching and isolating nitrifiers for penaeid hatchery systems were enriched primarily in Medium I by making 100-mL samples as the base of the medium. Meanwhile the filterent grains (1 g) of the biological filter set up from a prawn rearing system maintained in the laboratory were inoculated straight away in the Medium II for the primary enrichment. When the three pre enrichment systems with the water samples collected from the three locations as described above were conditioned, about 1g each filterent grains were removed from the filter bed and inoculated independently in Medium II. Also 1 mL each of the water sample was inoculated into Medium II and III.

The samples collected from low saline areas for isolating nitrifiers for non-penaeid hatchery system were divided in two lots. The water sample from a prawn grow out system at Maradu, Cochin, besides using for setting up Medium I was pre-enriched in a biological filter also. When the biological filter set up with water from prawn grow out system got conditioned, filterent grains and water samples from the filter set up were inoculated into Media II and III. The water sample from the biological filter of prawn larval rearing system at Shertallai was inoculated directly to Medium I and water sample and filterent grains were separately inoculated to Medium II and III. Likewise water sample and filterent grains from the biological filter of *Macrobrachium rosenbergii* rearing system in the laboratory was also inoculated into Medium I, II and III.

All primary enrichments were done as 100 mL aliquots in 250 mL conical flasks by incubating in room temperature on a rotary shaker at 100 rpm under obscurity. They were monitored daily for disappearance of NH_4^+-N and appearance of NO_2^--N with a drop in pH in the case of ammonia oxidizers, disappearance of NO_2^--N and building up of NO_3-N with apparently no marked pH change in the case of nitrite oxidizers. Replenishment of substrate and adjustment of pH were done regularly.

When growth of the cultures under primary enrichment attained near stationary phase marked with cessation in substrate consumption, a comparison of the cultures with respect to the total quantity of substrate consumed over a period of 231 days was also made. On the basis of lag experienced in each case, segregation of the most potent culture was made from each sampling station/site and was subjected for the secondary enrichment.

Secondary enrichment was done by inoculating 1 mL of the culture into freshly prepared respective media (Medium II). Subsequently they were maintained over magnetic stirrer at 100 rpm by adjusting the pH to 8 and adding fresh increments of the substrate as and when it got exhausted. The potentials expressed by these secondary enrichment cultures were assessed over a period of time (231 days). These cultures were subjected for a tertiary enrichment by inoculating 1 mL aliquots of the culture into freshly prepared Medium II and maintaining them in the same way as explained above. Meanwhile, the entire primary and secondary enrichment cultures and the four biological filters were also maintained by adding the substrate regularly and by adjusting the pH.

From non-penaeid hatchery system altogether 15 consortia of ammonia oxidizers and 15 of nitrite oxidizers were generated by the primary enrichment. All of them were subjected to a secondary enrichment by inoculating 1 mL of the

culture to the corresponding fresh media and the flasks were maintained in dark on a rotary shaker.

2.2.5 Selection of the best consortia

For penaeid hatchery system, the four ammonia oxidizing cultures and four nitrite oxidizing cultures were further subjected to an assessment to pick one culture each from each set based on their performance over a short period. For this, the tertiary enrichment cultures were charged with 10 μg mL^{-1} NH_4^+-N and 10 μg mL^{-1} NO_2^--N respectively and the consumption of the substrate for a period of 24 hours was determined. The culture, which consumed the maximum quantity of substrate, was segregated for amplification. Thus one ammonia oxidizer and one nitrite oxidizer was selected for penaeid hatchery system.

All the 15 consortia of ammonia oxidizers and 15 consortia of nitrite oxidizers of non-penaeid hatchery system were also assessed like this and one consortium of ammonia oxidizer and one consortium of nitrite oxidizer were selected for amplification.

2.2.6 Amplification of selected consortia in fermentor

From among the four consortia of ammonia oxidizers (AMOPCU 1, AMOPCU 2, AMOPCU 3, and AMOPCU 4) selected for penaeid hatchery system, AMOPCU 1 was segregated for further amplification. For nitrite oxidizers intended for Penaeid hatchery system, NIOPCU 1 was selected from among NIOPCU 1, NIOPCU 2, NIOPCU 3 AND NIOPCU 4. Likewise for non-penaeid hatchery system from among AMONPCU 1, AMONPCU 2 and AMONPCU 3; AMONPCU 1 was the ammonia oxidizer selected and NIONPCU 1 was the nitrite oxidizer selected from NIONPCU 1, NIONPCU 2 and NIONPCU 3.

The amplification of the consortia was carried out in a 2-L capacity New Brunswick Fermentor (BIOFLO 2000). Uniformly for all the four consortia, the medium according to Watson (1965) which consisted per 100 mL seawater (30 ppt for penaeid and 15 ppt for non-penaeid), 10 μg NH_4^+-N and 2 μg PO_4-P with a pH of 8, was used. The fermentor vessel along with the media was autoclaved at 10 lbs for 10 minutes. pH was adjusted to 8, temperature was set at 28^0C, agitation at 200 rpm and aeration at the rate of 0.6 L min^{-1} was provided. The fermentor culture vessel was covered with black cloth to protect the culture from light inactivation.

Initial NH_4^+-N and NO_2^--N in the media was noted. From the tertiary enrichment culture (in the case of ammonia oxidizers) and from the secondary enrichment culture (in the case of nitrite oxidizers), 1 % inoculum (v/v) was given to the fermentor. In the case of ammonia oxidizers as and when pH drop was noticed it was adjusted to 8 by automatic addition of 10 % sterile Na_2CO_3 through the base addition port. For nitrite oxidizers, pH drop did not occur.

Once in every 24 hours, the substrate uptake and product formation was monitored. When the substrates were consumed, exponential addition of the substrate was done aseptically through the substrate addition port. When the rate of substrate uptake and product formation declined, indicating the attainment of stationary phase, the culture was harvested and concentrated by centrifugation at 8000 rpm for 20 minutes at 4^0C in a cooling centrifuge (Remi). The centrifugate after washing with fresh medium was resuspended in corresponding medium containing 10 μg mL^{-1} substrate. This was stored in tightly stoppered sterile glass saline bottles, at 4^0C after determining the biomass, and from which cell yield and yield coefficient were worked out.

2.2.7 Primary characterization

For the primary characterization of the selected four consortia, a thin smear of the concentrated culture was made on microscope slide and subjected for Gram's staining. By Grams reaction, cellular morphology and zoogloeal formation, were observed.

2.2.8 Optimum growth conditions

For the successful development of the bioreactor technology for nitrifying hatchery water, mass production of the nitrifying bacterial consortia in fermentor is absolutely essential. When one goes for commercial production of the cultures the time required for the mass production has to be made minimum with high product yield. This can be made possible only if the generation of nitrifiers is made under optimum growth conditions. Therefore, a series of experiments were carried out to unravel the optimum substrate concentration, pH, temperature and salt content (Salinity $^{0}/_{00}$) required by the consortia for maximum substrate uptake and product formation and consequently the biomass generation over a period of time.

For all the consortia the medium according to Watson (1965) having the basic composition per 100 mL (either 30 ppt/15 ppt seawater) 10 μg NH_4^+-N / NO_2^--N and 2 μg PO_4-P with pH adjusted to 8 was used.

2.2.8.a pH

For the determination of optimum pH 50 mL aliquots of the medium in 250 mL conical flask, with the pH adjusted to 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9 using either 1 N HCl or 10 % Na_2CO_3 were used. For each pH four flasks were maintained and an aliquot of 0.5 mL inoculum was introduced from the amplified stock culture. Immediately after inoculation the content of NH_4^+-N and

NO_2^--N in the case of ammonia oxidizing consortium and the content NO_2^--N and NO_3-N in the case of nitrite oxidizing consortium were determined which served as the zero hour reading. Henceforth, at every 24-hour interval the substrate consumed and the product formed were monitored along with adjustment of pH to the original level. This process was continued till the substrate in any one of the sets of flasks got exhausted or consumed with almost the same quantity of the product formation. Based on the percentage consumption of NH_4^+-N and NO_2^--N, and percentage production of NO_2^--N and NO_3-N, the optimum pH of each consortium was determined.

2.2.8.b Temperature

On selecting the optimum pH, the experiment for optimum temperature was executed by maintaining uniformly the selected optimum pH in all the flasks and varying the temperatures as 4^0C, 20^0C, 28^0C, 37^0C and 45^0C. Substrate maintained was 10 μg mL^{-1} with the salinity at 30 and 15 ppt with respect to the type of consortia used. Based on the percentage consumption of NH_4^+-N and NO_2^--N and percentage production of NO_2^--N and NO_3-N, the optimum temperature for each consortium was determined.

2.2.8.c Substrate concentration

After selecting the optimum pH and temperature, the experiment for optimum substrate concentration was executed following the same line of protocol. For every consortium, corresponding optimum pH and temperature were setup with the substrate concentrations at 10, 50, 100, 150, 200, 250 and 300 μg mL^{-1}. Salinity in the flask was maintained according to the type of consortium used either at 30 ppt or 15 ppt. Based on the consumption of NH_4^+-N and NO_2^--N and production of NO_2^--N and NO_3-N for ammonia oxidizing and nitrite oxidizing consortia, the optimum substrate concentration required for each consortium was worked out.

2.2.8.d Salinity

Based on the optimum pH, temperature and substrate selected for each consortium, the next set of experiments to reveal their optimum level of salinity requirement was programmed. In this experiment the medium for each consortium was prepared in seawater diluted to get a range of salinity such as 10, 15, 20, 25, 30 and 35. Under these conditions the maximum substrate uptake and product formation were recorded to obtain the salinity preference by each consortium.

2.2.9 Maintenance of consortia

All the four cultures of ammonia oxidizers and nitrite oxidizers for penaeid and non- penaeid culture systems were amplified in fermentor. They were centrifuged, washed with medium and resuspended and maintained in fresh. Watson I (1965) medium with a substrate concentration of 10 μg mL^{-1} NH_4^+-N for ammonia oxidizers and 10 μg mL^{-1} NO_2^--N for nitrite oxidizers, and 2 μg mL^{-1} PO_4-P prepared per 100 mL of 30 or 15 ppt salinity sea water as the case may be. All cultures were stored in refrigerators at 4^0C. Periodic estimation of substrate concentration was done and substrate additions were done as and when it got exhausted so as to maintain a substrate concentration of 10 μg mL^{-1}.

2.2.10 Unit nitrifying activity

Unit nitrifying activity (UNA) of a nitrifying consortium is defined as the quantity of nitrifying biomass which can bring about the generation of 1 μ mole NO_2^--N min^{-1} L^{-1} in the case of ammonia oxidizers (UNAa) and the consumption of 1 μ mole NO_2^--N min^{-1} L^{-1} in the case of nitrite oxidizers (UNAn) under optimum conditions (Ramachandran, 1998). This is the most appropriate method of quantifying the potential of any nitrifying consortium as all other modes fail to

enable one to evaluate the activity of a consortium and to assess the quantity which should be used to charge a reactor for obtaining the expected activity.

The unit nitrifying activity of the consortium AMOPCU 1 and NIOPCU 1 were determined as described below: for the consortium AMOPCU 1 Watson (1965) medium containing 10 μg mL^{-1} NH_4^+-N having salinity 25 ppt and pH 7 was used. Aliquots of 99 mL, 95 mL, 90 mL and 85 mL medium were autoclaved in 500 ml screw capped saline bottles and inoculated with 1 mL, 5 mL, 10 mL and 15 mL consortium and the substrate concentration was adjusted to 10 μg mL^{-1}. The preparation was incubated in a serological water bath at 30 ^{0}C and air at the rate of 1 L min^{-1} was passed through, from a compressor. Starting from the zero hour of incubation, once in 12 hours, generation of NO_2^--N was estimated for 72 hours and the quantity of NO_2^--N production was worked out and expressed in μ mole NO_2^--N produced per minute per litre. For the consortium NIOPCU 1 medium according to (Watson, 1965) prepared in 25 ppt seawater, with pH adjusted to 7 and the substrate concentration made up to 10 μg mL^{-1} was used. The experimental setup was as described above and the preparation was incubated at 30^{0}C with air passed through at the rate of 1 L min^{-1}. The extent of consumption of NO_2^--N was worked out for a period of 72 hours and the NO_2^--N consumed was expressed as μ mole NO_2^--N consumed per minute per litre.

The unit nitrifying activity of AMONPCU 1 and NIONPCU 1 were determined as given below: for AMONPCU 1 Watson's medium containing 10 μg mL^{-1} NH_4^+-N having salinity 10 ppt, and pH 8.5 was used at a temperature of 45^{0}C. The extent of production of NO_2^--N was worked out and was expressed as μ mole NO_2^--N consumed per min per litre.

For NIONPCU 1, the optimum pH obtained was 4, which posed problems during large-scale production and usage. Therefore instead of this culture, the nitrite oxidizer (NIOPCU 1) developed for penaeid hatcheries was acclimatized to 15 ppt and was used for all subsequent studies replacing NIONPCU 1 and was

designated as NIONPCU 1a. Unit nitrifying activity of the transformed culture was determined at 15 ppt, pH 7 and temperature 28^0C and expressed as μ mole NO_2^--N consumed per minute per litre.

2.3 Results and Discussion

2.3.1 Pre-enrichment in biological filters

Water samples collected from Kannamali, Maala and Aayiramthengu were pre-enriched in a biological filter set up (Fig 2.1) for the nitrifying bacteria meant for penaeid systems. Similarly for non-penaeid systems, water samples from Maradu, Cochin was also enriched in the same pattern. These four systems could be monitored regularly for the consumption of NH_4^+-N and the production NO_2^--N and NO_3-N . Accordingly the total NH_4^+-N consumed over a period of 160 days was estimated and summarized in Table 2.1. Meanwhile there were already existing biological filter facilities in the laboratory, the one associated with a penaeid prawn rearing facility and another one for that of *Macrobrachium rosenbergii*. Besides, another biological filter facility associated with a prawn rearing system in a private hatchery at Sherthallai also could be used. However, a systematic analysis of the pattern of consumption of NH_4^+-N in these three already existing systems could not be made. Nevertheless it was well evident from the results of the analysis of the pattern of consumption of NH_4^+-N carried out at irregular intervals, that they were well conditioned and found suitable to be used as the sources of nitrifiers.

This pattern of pre-enrichment was essential due to the fact that nitrifiers compared with heterotrophic organisms were present in aquatic environment in very low order of magnitude in terms of few cells per mL (Watson, 1965). Moreover MPN estimates indicated zero or very few population of nitrifiers in the marine environment. And, the nitrifiers grow at a very low pace compared to heterotrophs. The pre-enrichment sufficiently helps to enhance the population

size in the filterent grains of the biological filter. Bed of a biological filter provides greater surface area for the nitrifiers to attach to and colonize making them 100 times more plentiful on the sand grains then suspended in water (Kawai *et al* ., 1964 and Spotte, 1979). It has to be emphasized here that within a short period (two weeks) of pre-enrichment, nitrification could be detected in the biological filter assembly, which over a period of 160 days could consume a good quantity of NH_4^+-N by each system indicating the development of a sizeable population of nitrifiers.

2.3.2 Assessment of adhesion of nitrifiers on the sand grains of a biological filter

Scanning electron micrographs of sand grains collected from the above biological filters are presented in Figs. 2.2 and 2.3. Surfaces of the sand grains are found to be colonized with bacteria adhering to the surface, thus forming a biofilm. Bazin *et al.,* (1982) observed that adhesion allowed the cells to respond rapidly to changes in concentration and rate of supply of substrates. Cox *et al.,* (1980) demonstrated that slime materials, polysaccharide in nature, is observed surrounding the colonies attached to the substratum. This slime material appears to 'cement' cells to the surface and may offer protection from unfavourable environmental conditions. However, they are not sure about its involvement in the initial attachment process. From the observations made it is apparent that the process of pre-enrichment of nitrifiers in a biological filter configuration is a good method of enhancing the nitrifiers in a limited space facilitating easy enrichment and isolation.

Another added advantage of setting a biofilter for the enrichment and isolation of nitrifiers from seawater is that it is possible to maintain the filter for a long period by just adding sufficient quantity of ammonium sulphate periodically. According to Aleem and Alexander (1958) during prolonged use of the biological filter nitrate may accumulate in the system resulting in the inhibition of

nitrification. As a remediation measure, one third of the water has to be replaced with aged and autoclaved seawater having the same salinity. Besides, the pipeline air filter also has to be replaced with fresh sterile units periodically.

2.3.3 Selection of appropriate media and the best consortia for amplification

Selection of an appropriate medium for ammonia and nitrite oxidizers was performed during the primary enrichment stage itself. As summarized in Table 2.2. and 2.3, and Figs 2.4, 2.5, 2.6, and 2.7, the performance of each medium in terms of NH_4^+-N and NO_2^--N consumed over a period of 231 days in the case of nitrifiers for penaeid hatchery system and over a period of 100 days in the case of nitrifiers for non-penaeid hatchery system was compared and the one which showed highest cumulative consumption was segregated. Interestingly, it was observed that highest level of consumption was in the medium of Watson (1965), (Medium II), the simplest one incorporated in the programme which was prepared in either 30 or 15 ppt seawater supplemented with either NH_4^+-N or NO_2^--N and PO_4-P as the case may be. The secondary and tertiary enrichments were performed with these cultures employing the same medium.

The extent of growth attained by four consortia of ammonia and nitrite oxidizers, meant for penaeid system, in the selected medium according to Watson (1965), was compared between each other as shown in Figs 2.8 and 2.9. In the same way performance of three consortia of ammonia and nitrite oxidizers meant for non-penaeid system was also assessed and summarized in Figs 2.10, 2.11, 2.12 and 2.13. Based on their performance following selection of consortia were made.

2.3.3.a For Penaeid hatchery system

The extent of NH_4^+ -N and NO_2^--N consumed over a period of 231 days by ammonia oxidizing and nitrite oxidizing consortia developed by primary enrichment from water samples straightaway and from the filtrant grains after pre-enrichment in a biological filter are summarized in Figs 2.4 and 2.5. Among all the cultures developed, the consortia of both ammonia and nitrite oxidizers obtained from the filterent grains of the biological filters set as the pre-enrichment systems exhibited comparatively higher potential in terms of the quantity of NH_4^+-N and NO_2^- -N oxidized over a period of time.

During the secondary enrichment over a period of 231 days they consumed NH_4^+-N from 251.47 to 274.86 mg mL^{-1} and NO_2^- -N from 272.53 to 298.48 mg mL^{-1}. During tertiary enrichment the total consumption during the same period was 233.12 to 264.91 mg mL^{-1} for NH_4^+-N oxidizers and 210 mg mL^{-1} for NO_2^--N oxidizers (Figs 2.6 and 2.7). From the consortia of ammonia and nitrite oxidizers developed one each was segregated based on their comparative performance over a period of 24 hours as is summarized. Accordingly the ammonia and nitrite oxidizers developed from the penaeid culture system of the Model Shrimp Farm, Maala (ADAK) was found to be the most efficient one and they were segregated for amplification.

However, all the eight cultures were named as follows and maintained for further use:

Description	Code given for each consortium
1. Ammonia oxidizing consortium Developed from the Model shrimp farm, Maala (ADAK)	AMOPCU 1

2. Nitrite oxidizing consortium developed from the Model shrimp Farm, Maala (ADAK)	NIOPCU 1
3. Ammonia oxidizing consortium Developed from the penaeid culture system of Kannamali	AMOPCU 2
4. Nitrite oxidizing consortium developed from the penaeid culture system of Kannamali	NIOPCU 2
5. Ammonia oxidizing consortium developed from the penaeid culture system of Aayiramthengu	AMOPCU 3
6. Nitrite oxidizing consortium developed from the penaeid culture system of Aayiramthengu	NIOPCU 3
7. Ammonia oxidizing consortium developed from the biological filter of a penaeid prawn rearing system maintained in the laboratory	AMOPCU 4
8. Nitrite oxidizing consortium developed from the biological filter of a penaeid prawn rearing system maintained in the laboratory	NIOPCU 4

2.3.3.b For Non-Penacid hatchery system

The extent of NH_4^+-N and NO_2^--N consumed over a period of 100 days by ammonia and nitrite oxidizers developed by primary enrichment from the water samples straightaway and from the filtrant grains of a biological filter from these different regions are summarized in Figs 2.8 and 2.9. As demonstrated in the figures the differences between them in terms of their overall consumption is very narrow and therefore all of them were subjected for a secondary enrichment. The overall consumption of NH_4^+-N and NO_2^--N during primary and secondary enrichment over a period of 283 days and 66 days respectively are summarized in Figs 2.10 and 2.12, with the comparative performance of these consortia over a period of 24 hours in Figs 2.11 and 2.13. Based on these results, one consortium each from each region was segregated, named as follows and maintained.

Description	Code name given for each consortium
1. Ammonia oxidizing consortium developed from prawn grow out system of Maradu, Cochin	AMONPCU 1
2. Nitrite oxidizing consortium developed from prawn grow out system of Maradu, Cochin	NIONPCU 1
3. Ammonia oxidizing consortium developed from the biological filter of a prawn larval rearing system at Sherthallai, Alleppey	AMONPCU 2

4. Nitrite oxidizing consortium developed from the biological filter of a prawn larval rearing system at Sherthallai, Alleppey — NIONPCU 2

5. Ammonia oxidizing consortium developed from the biological filter of *Macrobrachium* rearing system maintained in the laboratory — AMONPCU 3

6. Nitrite oxidizing consortium developed from the biological filter of *Macrobrachium* rearing system maintained in the laboratory — NIONPCU 3

From the consortia thus developed AMONPCU 1 and NIONPCU 1 were used for amplifying in the fermentor and the rest were preserved at 4°C.

To sum up, 14 nitrifying consortia could be developed which formed the broad base for the nitrifying bioreactors to be developed. This included 4 ammonia and nitrite oxidizers each suitable for penaeid culture system and three ammonia and nitrite oxidizers each for non-penaeid culture system. For the current application following consortia were used:

1. AMOPCU 1
2. NIOPCU 1
3. AMONPCU 1
4. NIONPCU 1

They were further amplified in a fermentor and used for the development of bioreactors.

The above description points to the fact that out of the three media tried, the one with the simplest composition performed well and among the various samples used, filterent grains from the conditioned biological filter set up as the pre-enrichment system contributed the best nitrifying consortia for further use. Selection of the media made here in the context of commercialization of the bioreactor technology assumes paramount importance, as the cost of production of the consortia required for immobilization in the bioreactor can be maintained very minimal. Henriksen (1981) is found to have used seawater enriched with NH_4Cl (0.5 mmol L^{-1}) and KH_2PO_4 (0.1 mmol L^{-1}) for the enrichment and cultivation of nitrifying bacteria from marine environment.

The filterent grains of the pre-enrichment system (conditioned biological filter) was found to be the best source of nitrifiers as the highly potent consortia were obtained from them. As the nitrifiers have been derived from the biofilm formed around the filterent grains, it strongly suggests that the property of forming biofilms on solid substratum can be taken advantage of, during the development of nitrifying bioreactors, as the ones envisaged here are biofilm reactors and the nitrifiers have to be immobilized on substratum for the formation of biofilms. The property of attachment to the filterent grains has helped substantially to device an appropriate and cost effective method of immobilization on a solid substratum as described in the succeeding chapters.

2.3.4 Amplification of the selected consortia

Amplification of consortia at this stage was carried out to generate sufficient nitrifying biomass for further experiments. When the consortia were grown under uncontrolled conditions the growth rate was very low and seldom resulted in turbid cultures. To control the required growth conditions and to get a higher biomass, a fermentor was used.

Pattern of growth obtained by the four selected consortia in the fermentor as batch process is depicted in Figs 2.14 to 2.17. Besides the overall substrate uptake, product formed, biomass generated, and the total number of days required for attaining stationary phase are summarized in Table 2.4. In the consortium AMOPCU 1 (Fig 2.14) production of NO_2^--N almost ceased with 20 days of incubation and hence forth, even though NH_4^+-N consumption was progressing exponentially NO_2^--N generation was found to be almost stagnant. It was apparent that the consumption of NH_4^+-N, which took place after 20 days incubation, was due to growth of heterotrophic component of the consortium. It may be suggested that 20 days shall be fixed as the time limit for terminating the amplification of the consortium.

In the case of NIOPCU 1 the NH_4^+-N consumption and the product (NO_2^--N) formation were almost in the same order of magnitude indicating minimal heterotrophic growth. The total biomass generated was about 119 mg over a period of 53 days. (Fig. 2.15)

The consortium AMONPCU 1 also exhibited the same pattern of NH_4^+-N consumption and NO_2^--N production, both having almost the same order of magnitude. The consortium attained stationary phase within 37 days and may be because of that the total quantity of NH_4^+-N consumed was comparatively lesser. For the same reason the biomass output happened to be comparatively lower (Fig 2.16)

In the case of NIONPCU 1 the overall biomass output obtained over a period of 48 days was comparatively higher (148 mg) and the pattern of consumption of NO_2^--N and production of NO_3-N was almost uniform indicating lesser heterotrophic growth (Fig. 2.17)

Except in the case of AMOPCU 1 there was more or less uniformity in the pattern of consumption of NH_4^+-N and NO_2^--N and the formation of NO_2^--N and

NO_3-N as the case may be. The stationary phase in nitrification was fixed when the rate of nitrification started getting reduced as demonstrated in the figures above. If the amplification continues, there is all likelihood to have more heterotrophic growth than that of nitrifiers and this will in turn lead to the generation of poor quality consortium with comparatively lesser nitrifiers.

2.3.5 Primary characterization

Gram stained preparations of the above four consortia are given in Figs 2.18 to 2.21. As clearly seen, the cells are embedded in a mucilaginous matrix known as glycocalyx, a product of bacteria itself composed of polysaccharides. Along with the majority of Gram negative rods, Gram positive bacteria also could be seen indicating that they were mixed culture consortia. In enrichment cultures ammonia oxidizing bacteria frequently occur as cell aggregates referred to as zoogloea or cysts. Zoogloea contains loosely associated cells embedded in a soft slimy layer while a cyst contain closely packed and compressed cells firmly embedded in and surrounded by a tough layer. According to Watson *et al.*, (1981) such aggregates are seldom seen in pure cultures. Nitrification is faster in mixed culture state than in pure form (Steinmuller and Bock, 1976). Therefore, it was decided to maintain the consortia as such without being resolved or purified considering that for all practical purposes they can be used as separate entities having unitary characteristics.

2.3.6 Optimum growth requirements

Performance of the consortia AMOPCU 1, NIOPCU 1, AMONPCU 1 and NIONPCU 1 in varying pH, temperature, substrate concentration and salinity in term of NH_4^+-N consumed and NO_2^--N produced for ammonia oxidizers and NO_2^--N consumed and NO_3-N produced for nitrite oxidizers are summarized in Tables 2.5 to 2.8. Selection of the optimum growth requirement was made based

on the highest NH_4^+-N uptake and NO_2^--N production for ammonia oxidizers and highest NO_2^--N uptake and NO_3^--N production for nitrite oxidizers with lowest standard deviation. Thus for AMOPCU 1, pH 7.5, temperature 28^0C, substrate concentration of 10 μg mL^{-1} and 30 ppt salinity was found to be the optimum requirements, while for NIOPCU 1 pH 7, temperature of 37^0C, substrate concentration 10 μg mL^{-1} and salinity of 25 ppt were found to be the optimum requirement. The ammonium oxidizing consortium meant for non-penaeid culture systems, AMONPCU 1 has optimum requirements like this: pH 8.5, temperature 28^0C, substrate concentration 10 μg mL^{-1} and salinity 10 ppt while the nitrite oxidizing consortium NIONPCU 1 has optimum requirements like this: pH 4.0, temperature 45^0C, substrate concentration 10 μg mL^{-1} and salinity 5 ppt.

Among the four consortia under study pH optima varied between 7.0 to 8.5 in the case of three consortia such as AMOPCU 1, NIOPCU 1 and AMONPCU1 and surprisingly the optimum pH of NIONPCU 1 was 4.0. According to Watson *et al.*, (1981), most strains of nitrifying bacteria grow optimally at a pH between 7.5 and 8. According to a study conducted by Engel and Alexander (1958), the pH curves of nitrifying bacteria are relatively flat between pH 7 and 9 and there is 50% reduction in activity when the pH falls to 6.2 or raise to 9.6. Meyerhof (1917), in another study demonstrated sharp increase in activity when the pH rise to 8.6 and the activity just gets reduced by 50% when the pH turns out to be 7.9 and 9.3. In this way reports on the ideal pH for nitrification vary much from culture to culture and place to place. However Kawai *et al.*, (1965), opined that the ideal pH for marine nitrifiers was 9 meanwhile according to Saeiki (1958), the optimum pH values for ammonia and nitrite oxidizing bacteria in sea water aquaria are 7.8 and 7.1 respectively. Srna and Baggaley (1975) determined that the marine nitrifiers used in their study operated best at pH 7.5 with an effective range at pH 7 to 8.2. Yoshida (1967) reported optimum values for four strains of unidentified marine ammonia oxidizers varying from 7 to 9.

In an experiment conducted by Forster (1974) the pH was lowered slowly and at pH 5.5 the nitrification stopped completely. In this context, determination of the optimum pH of NIOPCU 1 as pH 4 assumes much importance. Nitrifiers have been isolated from acid soil with pH 4 but several studies indicate that they cannot grow at pH less than 6. It has to be remembered that the consortium NIOPCU 1 was developed by enrichment in a medium where pH was maintained at 8.0 through out and the amplification had also been done at the same pH. The extreme pH of 4 as optimum was noticed only when it was experimentally determined with a consortium developed at pH 8. Such similar incidents could not be cited from literature. Meanwhile Ishaque and Cornfield (1974, 1976) found active nitrification in acid Bangladesh tea soils (pH 4.2) from which no chemoautotrophic nitrifiers could be isolated on a silica gel medium. Later they introduced samples of the English soil, rich in chemoautotrophic nitrifiers in the acid Bangladesh soil, but no stimulation of nitrification could be observed. However, with this experiment one cannot suggest that in acid soils nitrification is heterotrophic. This situation needs more investigation, as in Kerala, soils in almost all regions are acidic.

But in the present context the optimum pH 4 recorded with NIONPCU 1 does not favour in any way the requirement of a consortium for prawn larval rearing systems where the pH requirement is either neutral or slightly alkaline.

One mechanism by which pH affects the rate of nitrification has been proposed by Anthonisen (1974). This hypothesis is based on the fact that ammonia-ammonium and nitrite-nitrous acid equilibria depend on pH and alkaline pH favours the formation of free ammonia (NH_3) and free nitrous acid (HNO_2) which inhibit nitrifying organisms. According to him when the intracellular pH of nitrifying organisms is lower than the pH of the extracellular environment, free nitrous acid (FNA) permeates the cells and the nitrite ion. He again proposed that the ability of free ammonia (FA) and free nitrous acid (FNA)

to penetrate the nitrifying organism make them more inhibitory than ammonium and nitrite ions.

The optimum temperatures of 28^{0}C (AMOPCU 1 and AMONPCU 1), 37^{0}C (NIOPCU 1) and 45^{0}C (NIONPCU 1) recorded indicated the mesophilic nature of the consortia. In hatchery the rearing water has a temperature ranging from 25 to 30 ^{0}C and except that of NIONPCU 1 the temperature optima of all other consortia satisfy the requirement. The data further indicate that the nitrification proceeds better in warmer seasons or climates. Metcalf and Eddy (1973) pointed out that nitrification reaction follow the Vant Hoff Arhenius law upto 30^{0}C. As has been recorded here for one consortium, an optimum temperature of 42^{0}C has been recorded by Painter (1970) for a culture of *Nitrobacter* and Laudelout and Van Tichelen (1960), reported maximum growth of *Nitrobacter winogradskyi* at 42^{0}C and some growth upto 49^{0}C. The growth constants of nitrifying bacteria are affected greatly by temperature (Sharma and Ahlert, 1977). In experiments with marine nitrifying bacteria Watson (1965), found that cells incubated at 35^{0}C to 40^{0}C had a shorter generation time for the first five generations, but subsequently, the generation time was prolonged to 36 hours. Therefore 30^{0}C was proved to be the optimum temperature for growth.

According to Carlucci and Strickland (1968) one of the after effects of incubating nitrifiers at suboptimum temperatures is the prolonged lag experienced. In the development of nitrifying bioreactors such lag periods cannot be accommodated and in this context an optimum temperature determination for each consortium has very high significance.

Uniformly for all the four consortia 10 μg mL^{-1} was found to be the most ideal, after which, as the substrate concentration was increased the consumption of the substrate and the formation of the products of nitrification got diminished, indicating reduced nitrification potential. In natural environment ammonia and nitrite nitrogen never go up beyond 10 ppm and that is the case with hatchery

systems also. In the same way all ammonia oxidizers tested by Carlucci and Strickland (1968), were able to oxidize substrate at concentrations of ammonium found in seawater. Studies conducted by Srna and Baggaley (1975) in biological filter systems revealed that for a given initial concentration ranging from 0.035 to 1.96 g L^{-1} of NH_4^+-N, ammonia oxidation is a first order reaction as described in Michaelis-Menten kinetics. It implies that beyond 1.96 g L^{-1} NH_4^+-N inhibition of nitrification could take place. Eventhough similar comparable works could not be cited from literature with regard to nitrite oxidizers, Boon and Laudelout (1962) demonstrated that 1.4 g L^{-1} NO_2^--N caused 40 % inhibition in the activity of a pure culture of *Nitrobacter*. According to Anthonisen (1974) the degree of inhibition depends upon the ammonia-ammonium and the nitrite-nitrous acid equilibria which is pH dependent. Thus NH_4^+-N and NO_2^--N influence the nitrifying microorganisms in a double manner, one by being the main energy source and the other by being related to the concentration of inhibitory form as per the chemical equilibrium given below: (Anthonisen *et al*, 1976 and Fdz Polanco *et al.*, 1995)

$$NH_4^+ + OH^- \longleftrightarrow NH_3 + H_2O$$

$$2\,NO_2^- + 2\,OH^- \longleftrightarrow 2\,HNO_2$$

The determination 10 μg mL^{-1} as the optimum substrate concentration was taken advantage of in employing this concentration in the fermentors for the generation of nitrifying biomass. But as the biomass increases more substrate has to be added in small aliquots in such a way that the concentration per se does not go up beyond 10 μg mL^{-1}.

When the optimum salinity of the consortia was determined, those which were developed from marine environments (AMOPCU 1 and NIOPCU 1) preferred 30 and 25 ppt respectively and the one obtained from brackish water systems (AMONPCU 1 and NIONPCU 1) demonstrated optimum performance at 10 and 5 ppt. This agrees with the observation by Somville (1984) who says that

nitrifier populations from brackish water samples have optimum activity at a salt content slightly lower than that in the water where they live. Kawai *et al.*,(1965) reported that nitrifying activities in a warm sea water aquarium were greatest at the salinity to which the filter bed bacteria had been acclimated. Nitrifying activity diminished as the water was made more dilute or concentrated, although some activity remained even after the salinity was doubled. No activity could be detected after the filter bed organisms were immersed in fresh water. Srna and Baggaley (1975) demonstrated that decrease in salinity of 8 $^{0}/_{00}$ and an increase of 6 $^{0}/_{00}$ did not affect the rate of nitrification in aquarium seawater. This is especially true in the present context as nitrification could be detected in all salinities treated even though at lesser magnitude.

2.3.7 Maintenance of the consortia

The consortia generated in the fermentor were centrifuged at 8000 rpm and washed once with sterile medium and maintained at 4^{0}C in a refrigerator. Period of storage of each consortium and the viability afterwards are depicted in Table 2.9. It shows that even after prolonged storage the viability of the consortium could be maintained at 4^{0}C.

2.3.8 Unit nitrifying activity of the consortia

The unit nitrifying activity of the consortia is given in Table 2.10. The unit activity was worked out from the mean of substrate consumed and product formed with every inoculum size used ranging from 1 to 15 mL. Uniformly the unit activity was found to have diminished as the quantity of inoculum was increased. Thus it followed the Michaelis-Menten equation, which stated that, the activity of an organism was proportional to the substrate and as the inoculum size increased the substrate concentration also should be increased. By definition one unit nitrifying activity (UNA) of a nitrifying consortium is defined as the quantity of nitrifying biomass which can bring about the generation of 1 μ mole NO_2^--N min

L^{-1} in the case of ammonia oxidizers (UNAa) and the consumption of 1 μ mole NO_2^--N min L^{-1} in the case of nitrite oxidizers (UNAn) under optimum conditions. Here the optimum conditions were the ones already determined as the optimum pH, temperature, salinity and substrate concentration.

This sort of determination of the capability of nitrifying consortium is of high practical importance as it is essential to know how much inoculum in terms of quantity should be used for each reactor. Being a mixed culture consortium every batch of the culture will be having a particular nitrifying potential, which may vary from another batch even though prepared under the same conditions. By finding out the unit activity one can even predict the potential which can be expected out of a reactor on activation.

This method of quantifying nitrifying bacteria by its nitrifying potential has many advantages. Being a mixed culture consortium cell counts will not give the actual potency as it consists of heterotrophs also. In the case of MPN counts for nitrifiers even after 100 days of incubation the activity could not be detected as per the observations made by Matulewich *et al.*, (1975). Because of the low growth yield it is not possible to use mere absorbance at A_{600} as the measure of nitrifying biomass. Determination of production of NO_2^--N in the case of ammonia oxidizers and consumption of NO_2^--N in the case of nitrite oxidizers have been validated by Loveless and Painter (1968) who found that the greatest sensitivity, simplicity and accuracy of nitrite determination makes it a better choice than the estimation of cell carbon, mass or number. The pattern of consumption of NH_4^+-N cannot also be relied upon as being mixed culture consortium ammonia can be consumed as nitrogen source even by the associated heterotrophs. Production of NO_3-N also can not be much relied upon. The Hydrazine Sulphate reduction may not always give 100% reduction of nitrate to nitrite for its accurate determination.

As one step forward to make the process of determination of unit nitrifying activity simpler a special device has to be designed.

2.4 Summary

Nitrifying bacterial consortia required for immobilizing and activating bioreactors meant for penaeid and non- penaeid larval rearing system were developed by a series of pre primary, secondary and tertiary enrichment procedures. From 14 such consortia thus developed 4 were segregated, 2 ammonia and 2 nitrite oxidizing based on their potency. They were amplified in fermentor concentrated by centrifugation and maintained at 4°C. Optimum growth requirements of the consortia were determined. While doing so the consortium NIONPCU 1 was not found to be suitable for applying in prawn larval rearing system and therefore the consortium NIOPCU 1 was acclimatized to suit the requirements of non-penaeid systems, designated as NIONPCU 1a and used subsequently. Unit nitrifying activity of all the four segregated consortia were used for mass production and determination of the growth kinetics under optimum growth conditions in fermentor as described in the next chapter.

Table 2.1. Total NH_4^+- N consumed by the biological filters setup with water from Kannamaali, Maala, Aayiramthengu and Maradu over a period of 160 days.

Sample	**Salinity (ppt)**	NH_4^+- N consumed over a period of 160 days (mgL^{-1})
Kannamaali	30	185.31
Maala	30	206.54
Aayiramthengu	30	274.14
Maradu	15	89.06

le 2.2. Samples and sources of nitrifying consortia

For penaeid system.

o	Sampling station	Samples	Primary enrichment of the sample	Mode of pre enrichment	Primary enrichment of the pre enriched sample	Total no.of cultures developed per medium
	Penaeid grow out system at Kannamali Cochin	Water from the grow out system	Water sample in Medium I	Pre - enrichment in a biological filter	Water from the biofilter Medium II, MediumIII. Filterant grains from biofilter MediumII	M I : 2 M II: 4 M III:2 4+4
	Model shrimp farm and training centre Maala. Trichur (ADAK)	Water from the grow out system	Water sample in Medium I	Pre - enrichment in a biological filter	Water from the bio filter: Medium II and Medium III. Filterant grains from biofilter: Medium II	M I: 2 M II: 4 M III:2 4+4
	Penaeid grow out system at Ayiramthen gu	Water from the grow out system	Water sample in Medium I	Pre- enrichment in a biological filter	Water from the biofilter:MediumII and Medium III	M I: 2 M II: 4 M III:2 4+4
	Biological filter of a penaeid rearing system in the lab	Filterant grains from the biofilter	Filtrant grains in Medium II	No further pre-enrichment as the sample was from a biofilter	Not required	M II:2 1+1
						13 + 13 = 26

e :Total 13 consortia of ammonia oxidizers and 13 of nitrite oxidizers could be
ured at primary culture level.

2.3. Samples and Sources of nitrifying consortia.

B. For Non-penaeid system.

Sampling station	Sample	Primary enrichment of the sample	Mode of pre-enrichment	Primary enrichment of the pre - enriched sample	Total no. of cultures developed per medium
Prawn grow out system at Maradu , Cochin	Water from the grow out system	Water sample in Medium I	Pre enrichment in a biological filter	Water from biofilter : M II and M III Filterant grains from biofilter : MII and MIII	M I : 2 M II : 4 M III :4 5+5
Biological filter of a prawn larval rearing system at Sherthalai, Alleppey	Water and filterent grains	Water sample in Medium I, Water sample in Medium II and Medium III,Filterant grains in Medium II and Medium III	No further pre-enrichment as the sampling was from a biological filter	Not required	M I : 2 M II : 4 M III: 4 5+5
Rearing system for *Macrobrachium* with a biological filter set up in the lab	Water and filterant grains	Water sample in Medium I, Water sample in Medium II and Medium III,Filterant grains in Medium II and Medium III	No further pre-enrichment as the sampling was from a biological filter	Not required	M I :2 M II :4 M III : 4 5+5

t: From each source five consortia were developed for ammonia oxidizers and five :trite oxidizers. Total 15 consortia of ammonia oxidizers and 15 of nitrite oxidizers :be obtained at primary culture level.

Table 2.4. Substrate uptake, product formed, biomass generated, total number of days required for attaining stationary phase for the four consortia amplified in fermentor.

Code name of the consortium	Substrate uptake (mgL^{-1})	Product formed (mgL^{-1})	Biomass generated (mg dry weight L^{-1})	Total biomass generated in 2L fermentor (mg)	Total number of days required for attaining stationary phase
AMOPCU 1	1403.6297	292.384	65	130	27
NIOPCU 1	1976.2321	1775.21	59.4	118.8	53
AMONPCU 1	303.6489	421.0147	30	60	37
NIONPCU 1	441.8829	356.1668	73.75	147.5	48

Table 2.5. Performance of the consortium AMOPCU 1 in varying pH, temperature, substrate concentration and salinity in terms of NH_4^+-N consumed and NO_2^--N produced (n=4)

Performance in varying **pH**			Performance in varying **temperature**			Performance in varying **substrate Concentration**			Performance in varying **salinity**		
Varying pH	NH_4^+-N consumed µg.mL^{-1}	NO_2^--N produced µg.mL^{-1}	Varying **temperature (°C)**	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **substrate concentration µg. mL^{-1}**	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg.mL^{-1}	Varying **salinity (ppt)**	NH_4^+-N consumed µg mL^{-1}	% NO_2^--N produced from the NH_4^+-N consumed
4.0	7.89 ±6.9	0.36±0.6	**4**	7.97± 9.90	23.25±40.2	**♦10**	97.36+ 4.58	93.22+17.82	**10**	14.93±13.64	16.53± 21.56
4.5	0	0.17±0.3	**20**	64.54 ±14.5	184.6±11.2	**50**	34.69± 12.59	91.24±42.85	**15**	93.34±4.71	66.17± 11.79
5.0	0.99 ±1.7	1.3±2.26	**♦28**	83.27 +3.44	229.2+16.6	**100**	9.82± 10.04	1827.5± 1672	**20**	97.67±1.64	65.59± 6.74
5.5	2.12 ±2.2	25.6±27	**37**	48.41 ±13	751.0±280	**150**	9.45± 6.14	59.11±37.74	**25**	92.19±3.91	89.2± 14.2
6.0	80.2 ±5.3	159.8± 89	**45**	0.343 ±0.6	1772.6±3070.22	**200**	3.87± 5.08	64.03±82.33	**♦30**	97.05+1.97	78.26+ 1.93
6.5	20.4 ±16.6	589.7± 741.2				**250**	12.55± 5.06	54.83±12.77	**33**	93.41±3.53	83.45± 11.96
7.0	95.5 ±4.3	332.3± 14.1				**300**	2.89± 3.31	56.49±60.97			
♦7.5	99.4 +1.1	320+9 8.84									
8.0	97.3 ±1.7	169.04 ±106.2									
8.5	97.1 ±2.1	180.4 ± 20.3									
9.0	93.6 ±6.1	140.9± 16									

♦ Optimum selected

Table 2.6. Performance of the consortium NIOPCU 1 in varying pH, temperature, substrate concentration and salinity in terms of NO_2^--N consumed NO_3^--N produced (n=4)

Performance in varying **pH**			Performance in varying **temperature**			Performance in varying **substrate Concentration**			Performance in varying **salinity**		
Varying pH	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **temperature (°C)**	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **substrate concentration µg mL^{-1}**	NH_4^+-N consumed µgmL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **salinity (ppt)**	NH_4^+-N consumed µg mL^{-1}	% $NO2^-$ N produced µg mL^{-1}
4.0	98.8± 1.17	11.6± 20.0	**4**	12.2± 1.93	41.2± 37.2	**♦10**	100+0	131.4+ 14.44	**10**	82.45 ± 1.6	107.9± 9.72
4.5	93.1± 0.2	101± 30.4	**20**	47.5± 6.49	101± 15.85	**50**	24.66± 7.12	4.98± 8.63	**15**	89.15 ± 3.5	134± 13.97
5.0	77± 2.34	40± 30.7	**28**	94.4± 0.70	128± 7.17	**100**	41.9± 17.81	128± 151.74	**20**	97.74 ± 2.01	124.8± 6.42
5.5	36.4± 2.63	48.8± 11.9	**♦37**	99.1+ 0.31	120+ 5.58	**150**	36.58± 6.72	66.6± 70.11	**♦25**	100± 0	117.6± 10.33
6.0	99.9± 0.25	96.1± 16.6	**45**	97.22± 3.19	112± 5.57	**200**	17.91± 6.92	0	**30**	99.94 ± 0.102	115.5± 9.67
6.5	100±0	112± 21.7				**250**	16.98± 1.61	0	**33**	99.94 ± 0.11	125.7± 16.09
♦7.0	99.8± 0.12	109+ 7.15				**300**	13.18± 4.92	0			
7.5	99.9± 0.19	69.6± 46.6									
8.0	58.7± 41.3	54.6± 54.6									
8.5	100±0	110± 16.7									
9.0	100±0	122± 11.0									

♦ Optimum selected

Table 2.7. Performance of the consortium AMONPCU 1 in varying pH, temperature, substrate concentration, and salinity in terms of NH_4^+-N consumed, NO_2^--N produced. (n= 4)

Performance in varying pH			Performance in varying **temperature**			Performance in varying **substrate Concentration**			Performance in varying **salinity**		
Varyin pH	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **temperature(°C)**	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **substrate concentration µg.mL^{-1}**	NH_4^+-N consumed µg mL^{-1}	NO_2^--N produced µg mL^{-1}	Varying **salinity (ppt)**	NH_4^+-N consumed µgmL^{-1}	% $NO2^-$N produced µg mL^{-1}
4.0	0	0	**4**	50.30± 9.93	2.81±2.8	**♦10**	82.6± 4.8	109.8± 12.7	**♦10**	96.4± 0.6	143± 13
4.5	0.51± 0.9	0	**20**	60.89± 5.8	5.91± 1.3	**50**	35.2± 5.4	124± 22.81	**15**	58.6± 14.4	26.3± 19.17
5.0	0.002± 0.0033	0	**♦28**	97.97± 3.52	227± 25.89	**100**	55.8± 18.67	41.15± 11.04	**20**	39.05± 11.90	33.69± 16.46
5.5	0	0	**37**	70.1± 4.4	443.2± 121.4	**150**	25± 25	87.63± 52.87	**25**	31.14± 12.66	7.91± 3.89
6.0	17.26± 10.33	153.4± 94.79	**45**	98.44± 2.29	273.2± 67.75	**200**	13.3± 12.66	17.21± 12.69	**30**	36.4± 6.74	3.84± 1.56
6.5	18.36± 6.91	372± 150.8				**250**	7.33± 8.49	26.22± 30.61	**33**	41.44± 5.83	3.65± 0.26
7.0	13.30± 13.40	71.19± 71.19				**300**	4.78± 9.56	22.95± 45.94			
7.5	0.92± 1.59	256.4± 444.08									
8.0	25.26± 16.39	116.2± 89.64									
8.5	56.44± 13.40	63.69± 9.04									
9.0	68.15± 9.85	66.76± 17.21									

♦ Optimum selected

Table 2.8 Performance of the consortium NIONPCU 1 in varying pH, temperature, substrate concentration, and salinity in terms of NH_4^+-N consumed, NO_2^--N produced (n=4)

Performance in varying **pH**			Performance in varying **temperature**			Performance in varying **substrate Concentration**			Performance in varying **salinity**		
Varying **pH**	NO_2^--N consumed µg mL^{-1}	NO_3^--N produced µg mL^{-1}	Varying **temperature(°C)**	NO_2^--N consumed µg.mL^{-1}	NO_3^--N produced µg. mL^{-1}	Varying **substrate concentration** µg.mL^{-1}	NO_2^--N consumed µg.mL^{-1}	NO_3^--N produced µg.mL^{-1}	Varying **salinity (ppt)**	NO_2^--N consumed µg.mL^{-1}	NO_3^--N produced µg. mL^{-1}
♦**4.0**	81.3 ±8.6	103 ±21 .4	**4**	83.7± 2.8	53.9 ±12	♦**10**	97.8± 1.8	74.5± 8.4	♦**5**	99.9± 0.17	100± 5.93
4.5	8.6± 8.6	43. 8±5 6.2	**20**	94.8± 1.2	62.2 ±12	**50**	90.4± 4.5	61.1± 6.3	**10**	97.9± 1.1	77.6 ±5.3
5.0	0	0	**28**	97.2± 0.4	53.3 ±4.1	**100**	73.2± 15.2	65.3± 5.1	**15**	86.1± 2.4	76.8 ±11. 1
5.5	0	0	**37**	97.3± 0.3	62.2 ±4.3	**150**	72.9± 15.2	10.7± 18.6	**20**	84.4± 8.8	67.5 ±4.4
6.0	0	0	♦**45**	98.2± 0.8	70.3 ±9.7	**200**	51.7± 8.2	0	**25**	94.9± 7.1	69.8 ±11. 6
6.5	0	0				**250**	37.9± 13	0	**30**	95.4± 0.6	63.4 ±6.7
7.0	0	0				**300**	20.2± 12.3	0	**33**	789± 1.2	49.1 ±9.2
7.5	0	0									
8.0	0	0									
8.5	0	0									
9.0	0	0									

♦ Optimum selected

Table. 2.9. Period of storage of each consortium and viability

Code name of the consortium	Storage		Viability
	From	Dates on which viability was recorded	
AMOPCU 1	19-12-97	25-01-99 , 19-07-99	Viable
NIOPCU 1	20-04-98	05-11-98 , 04-03-99	Viable
AMONPCU 1	26-06-98	29-05-99	Viable
NIONPCU 1	29-08-98	24-09-99, 22-10-99	Non-viable

Table 2.10. Unit nitrifying activity of the four consortia amplified in fermentor

Code name of the consortium	Unit nitrifying activity (UNAn) Mean ± Standard deviation
AMOPCU 1	37.3353 ± 0.2989
NIOPCU 1	35.3589 ± 7.9172
AMONPCU 1	71.39 ± 0
NIONPCU 1 a (NIOPCU 1 acclimatized to 15 ppt)	13.9919 ± 0.9019

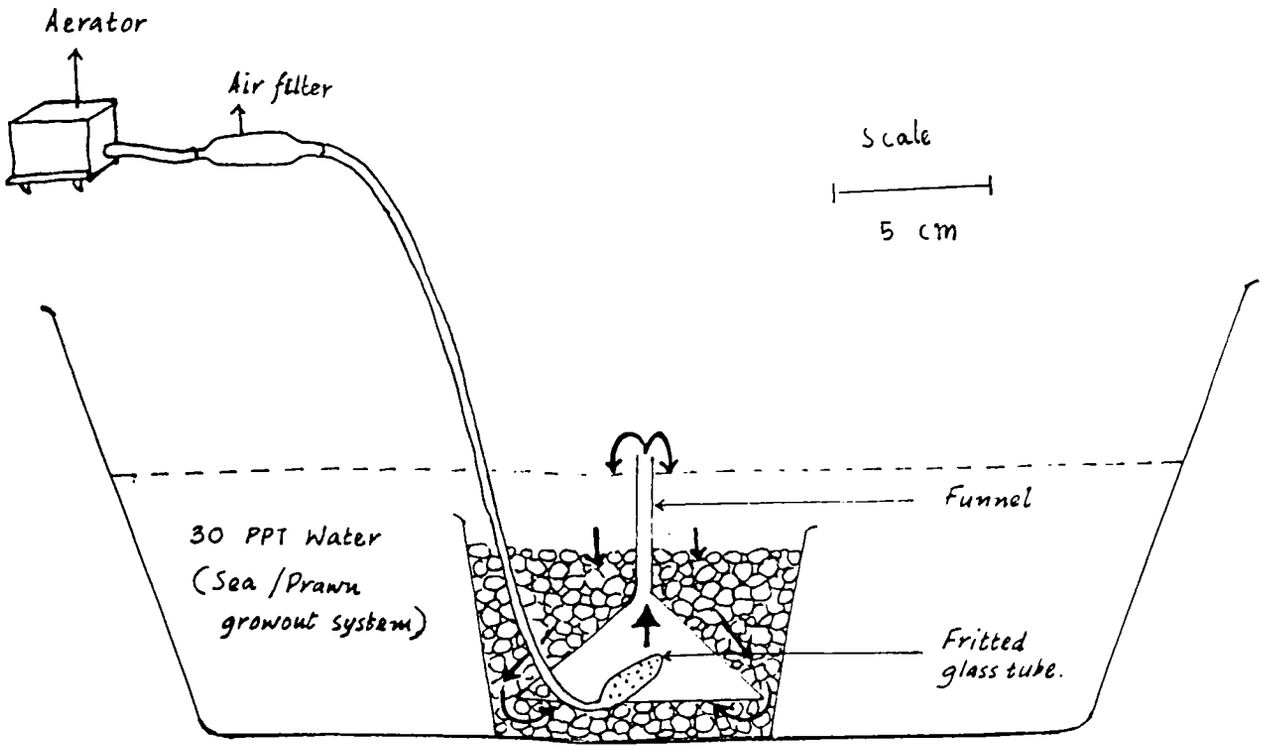
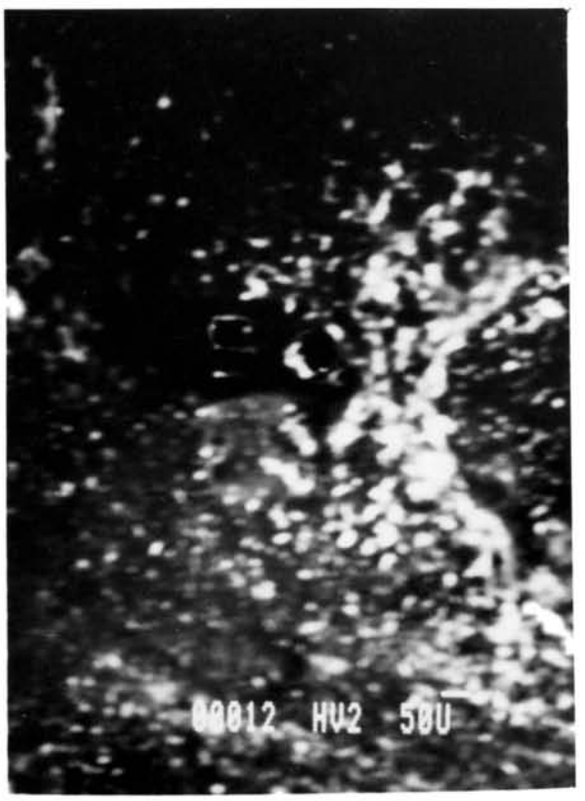


Fig 2.1 Biological filter setup for pre enrichment of nitrifiers

Fig 2.2 Scanning electron micrograph showing colonization of nitrifying bacteria on sand grains in the biological filter

Fig 2.3 Scanning electron micrograph showing colonization of nitrifying bacteria on grains in the biological filter


00012 HV2 50U


00006 HV2 50U

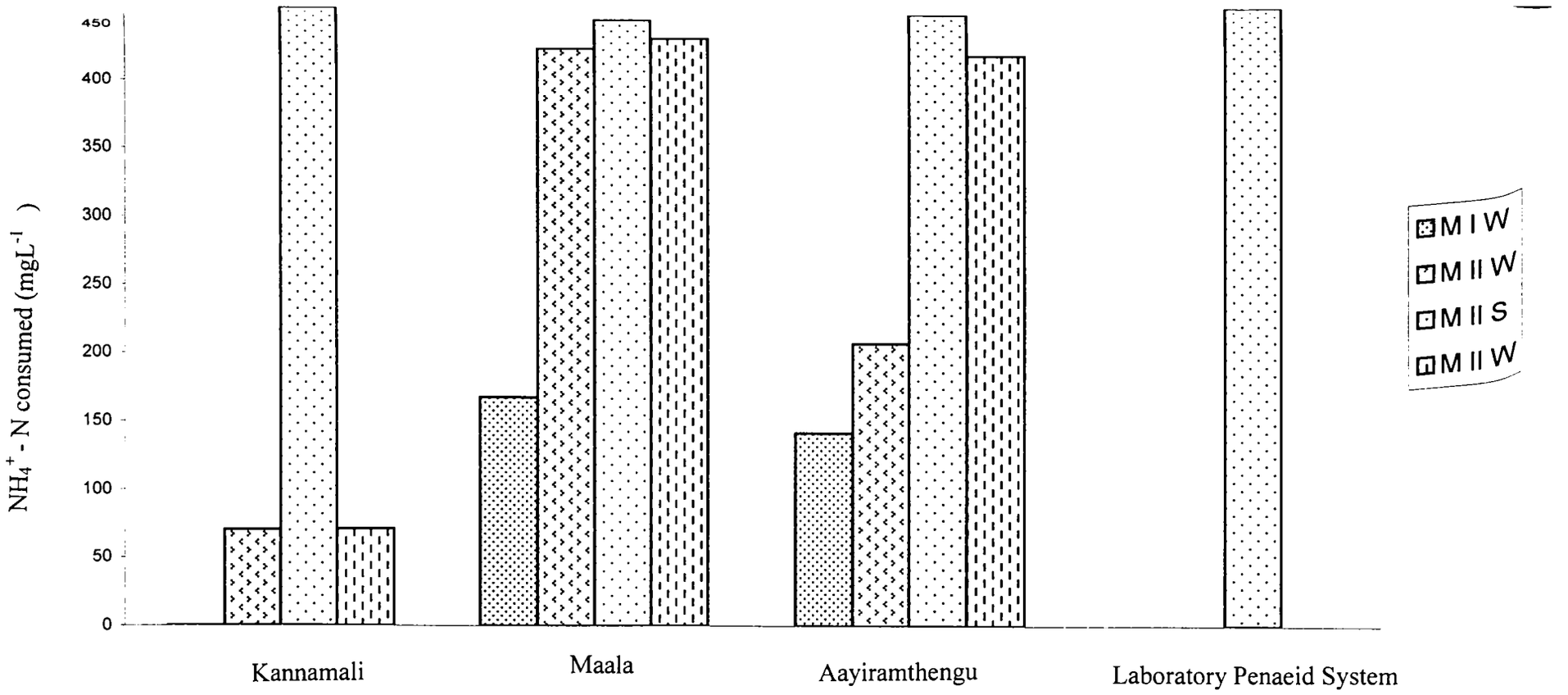


Fig 2.4 Comparison of the extent of growth attained by consortia of ammonia oxidizers in various media in terms of NH_4^+ - N consumed , enriched directly from water sample as well as through filterant grains of a biological filter (over 231 days) for penaeid hatchery system

(M I W- Medium I with water sample, M II W- Medium II with water from biological filter, M II S - Medium II with filterant grains from biological filter, M III W- Medium III with water from biological filter)

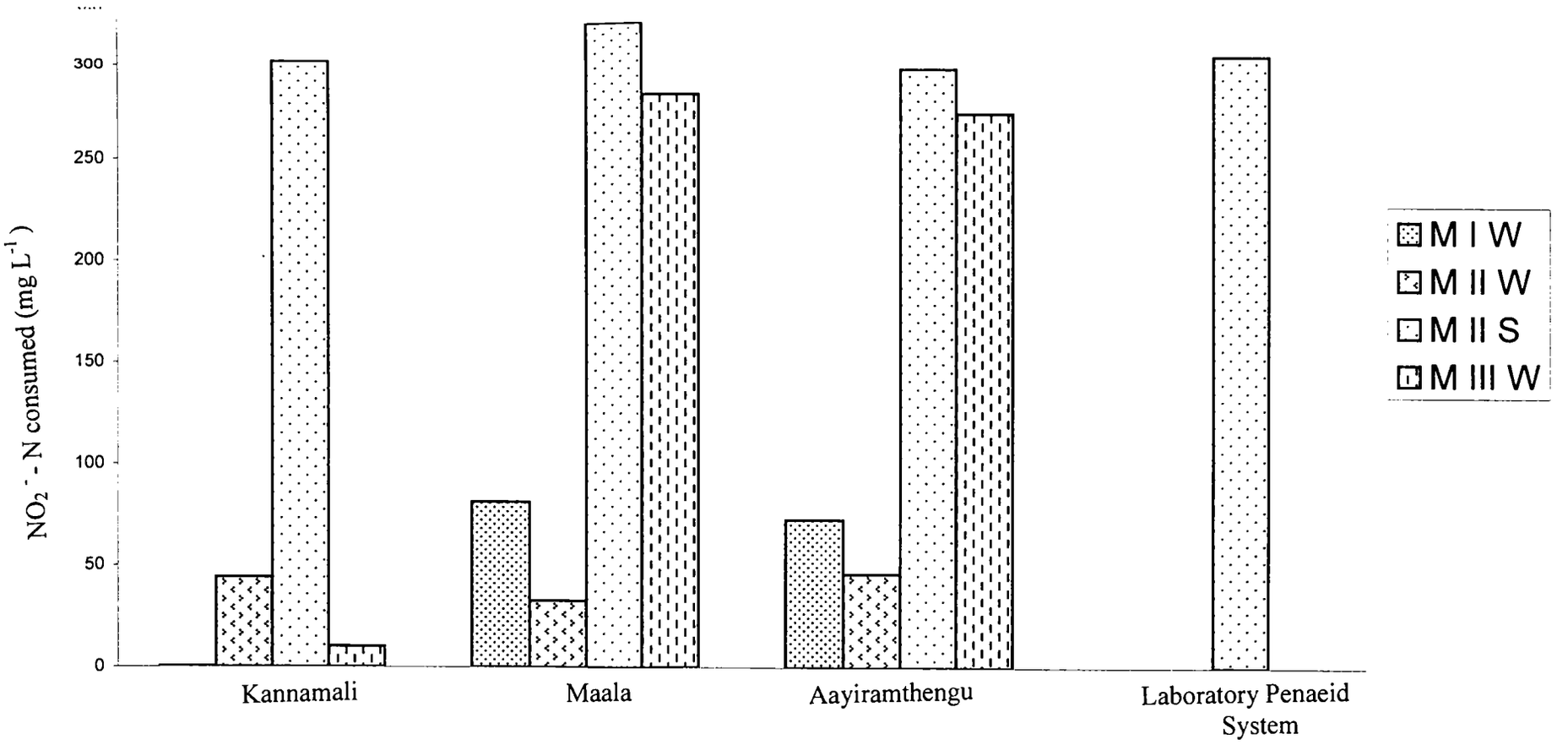


Fig 2.5 Comparison of the extent of growth attained by consortia of nitrite oxidizers in various media, in terms of NO_2^- - N consumed, enriched directly from water sample as well as through filterant grains of a biological filter (over 231 days) for penaeid hatchery system.

(M I W- Medium I with water sample, M II W- Medium II with water from biological filter, M II S - Medium II with filterant grains from biological filter, M III W- Medium III with water from biological filter)

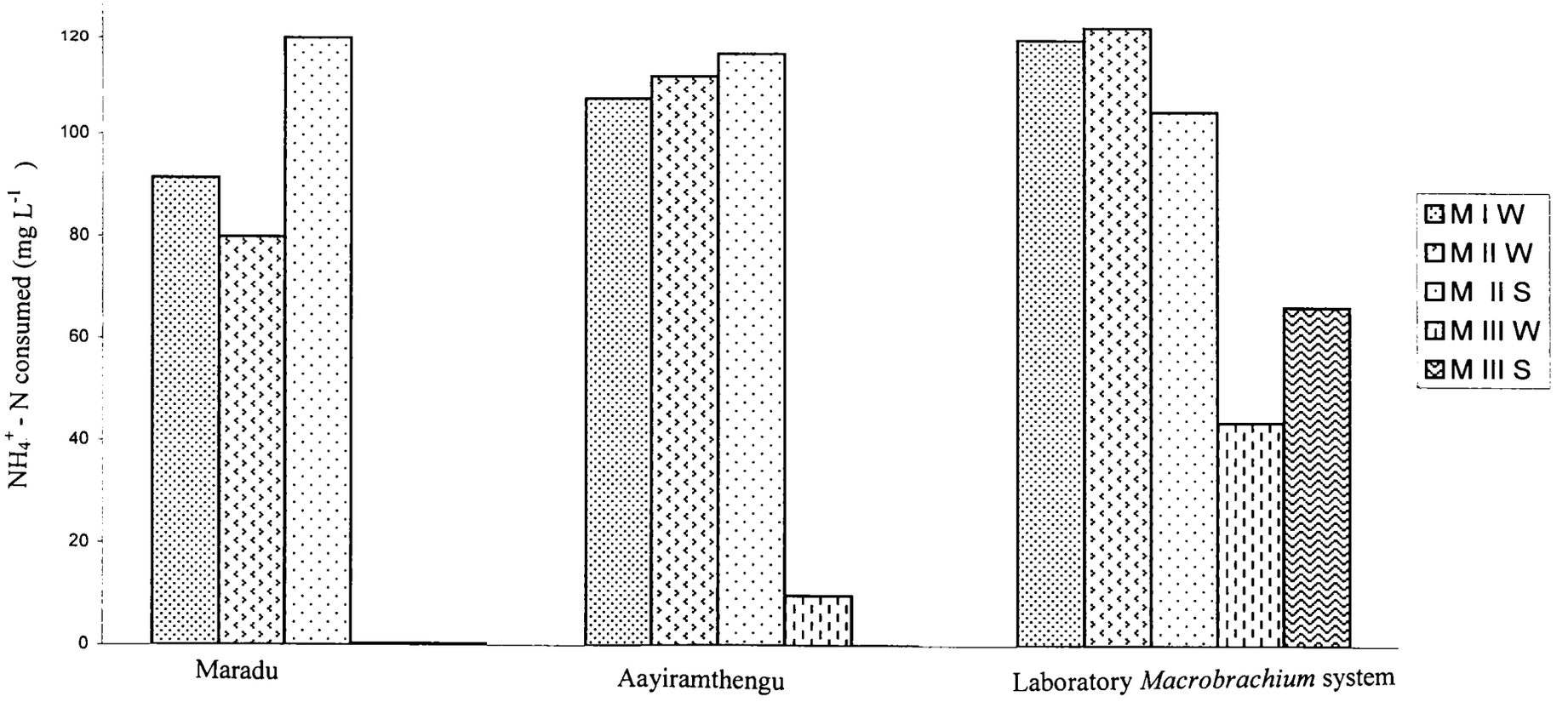


Fig 2.6 Comparison of the extent of growth attained by consortia of ammonia oxidizers in various media in terms of NH_4^+ - N consumed , enriched directly from water sample as well as through filterant grains of a biological filter (over100 days) for non-penaeid hatchery system

(M I W- Medium I with water sample, M II W- Medium II with water from biological filter, M II S - Medium II with filterant grains from biological filter, M III W- Medium III with water from biological filter, M III S - Medium III with filterant grains from biological filter)

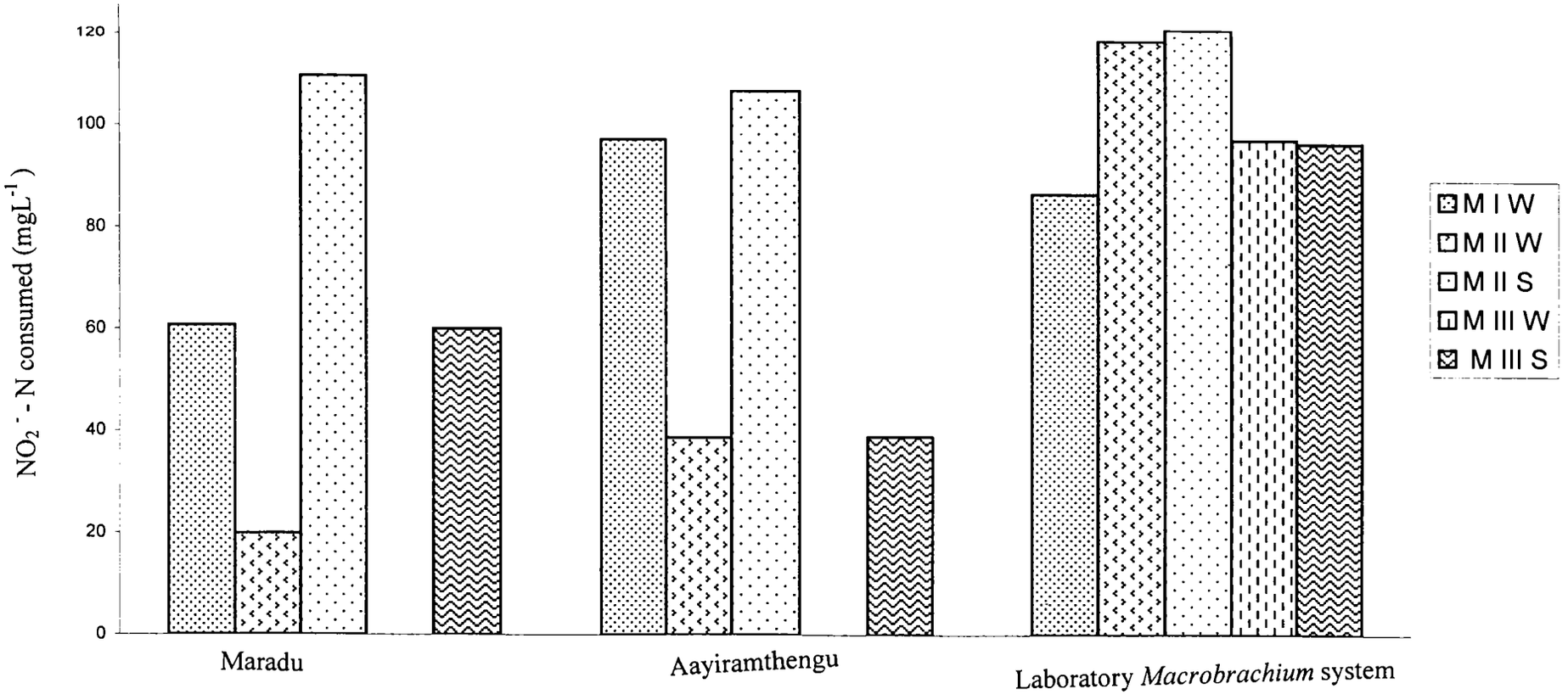


Fig 2.7 Comparison of the extent of growth attained by consortia of nitrite oxidizers in various media in terms of NO_2^- - N consumed , enriched directly from water sample as well as through filterant grains of a biological filter (over100 days) for non-penaeid hatchery system

(M I W- Medium I with water sample, M II W- Medium II with water from biological filter, M II S - Medium II with filterant grains from biological filter, M III W- Medium III with water from biological filter, M III S - Medium III with filterant grains from biological filter)

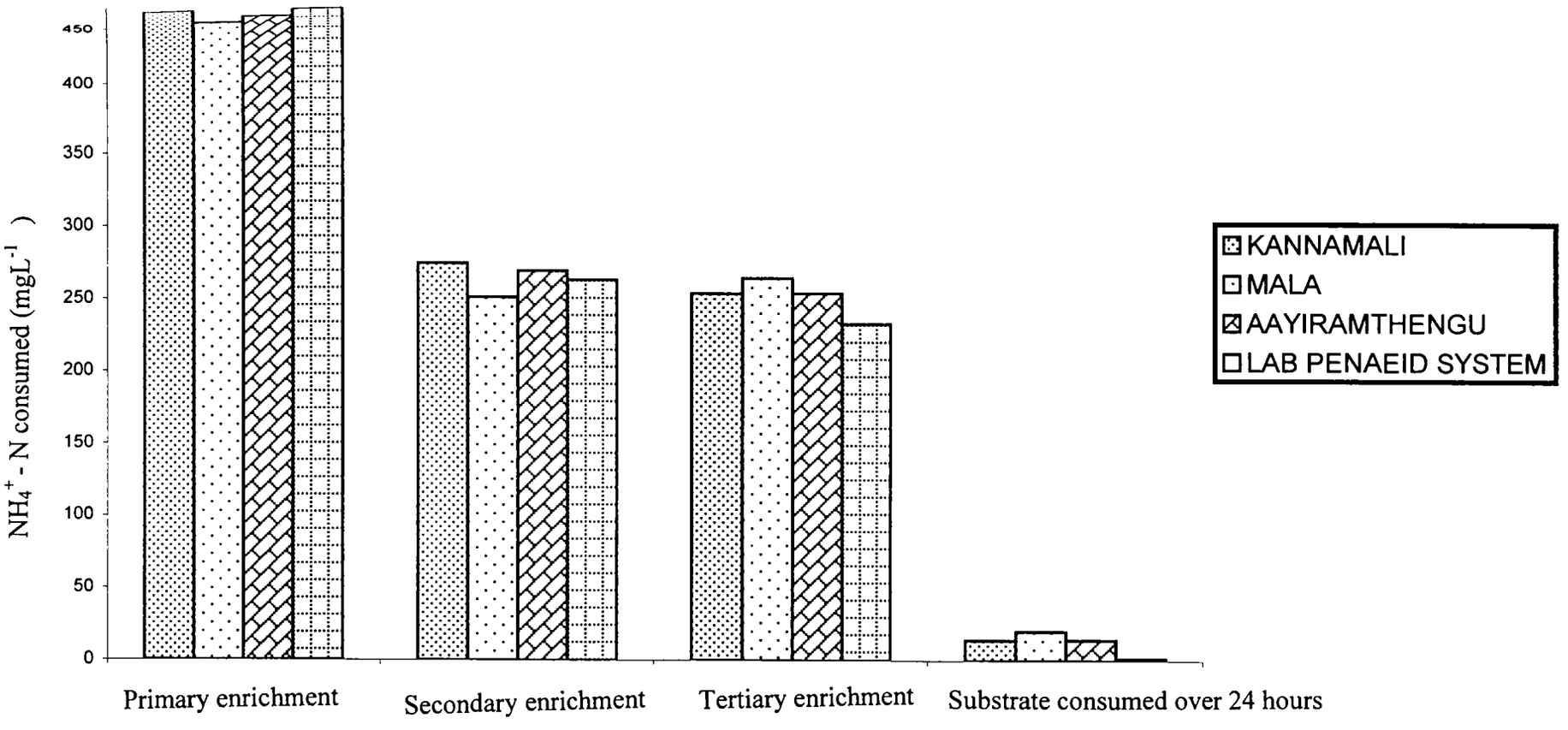


Fig 2.8 Comparison of the extent of growth attained by the selected consortia of ammonia oxidizers during primary, secondary and tertiary enrichment over a period of 231 days uniformly for penaeid hatchery system

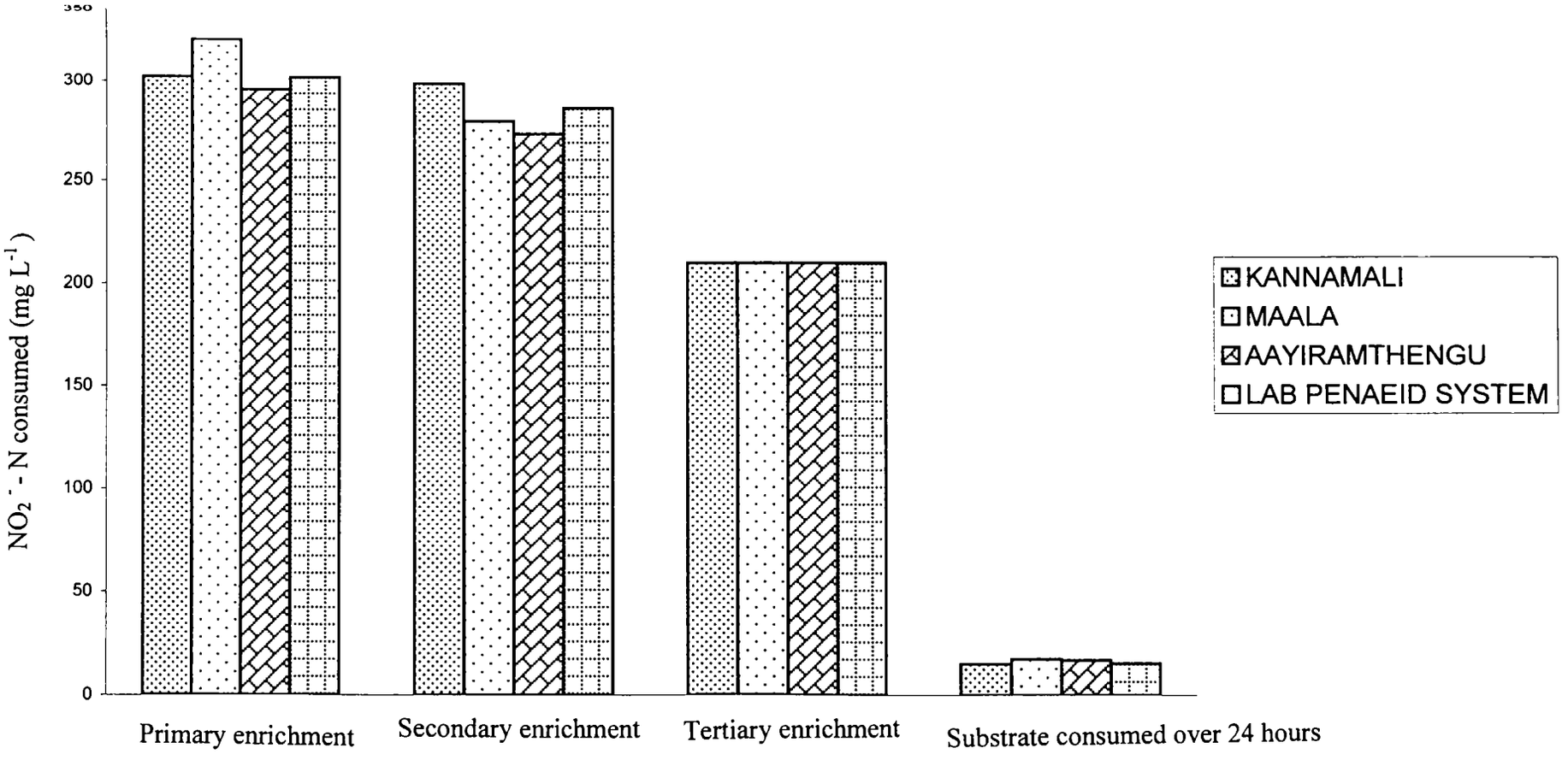


Fig 2.9 Comparison of the extent of growth attained by the selected consortia of nitrite oxidizers during primary, secondary and tertiary enrichment over a period of 231 days, uniformly for penaeid hatchery system

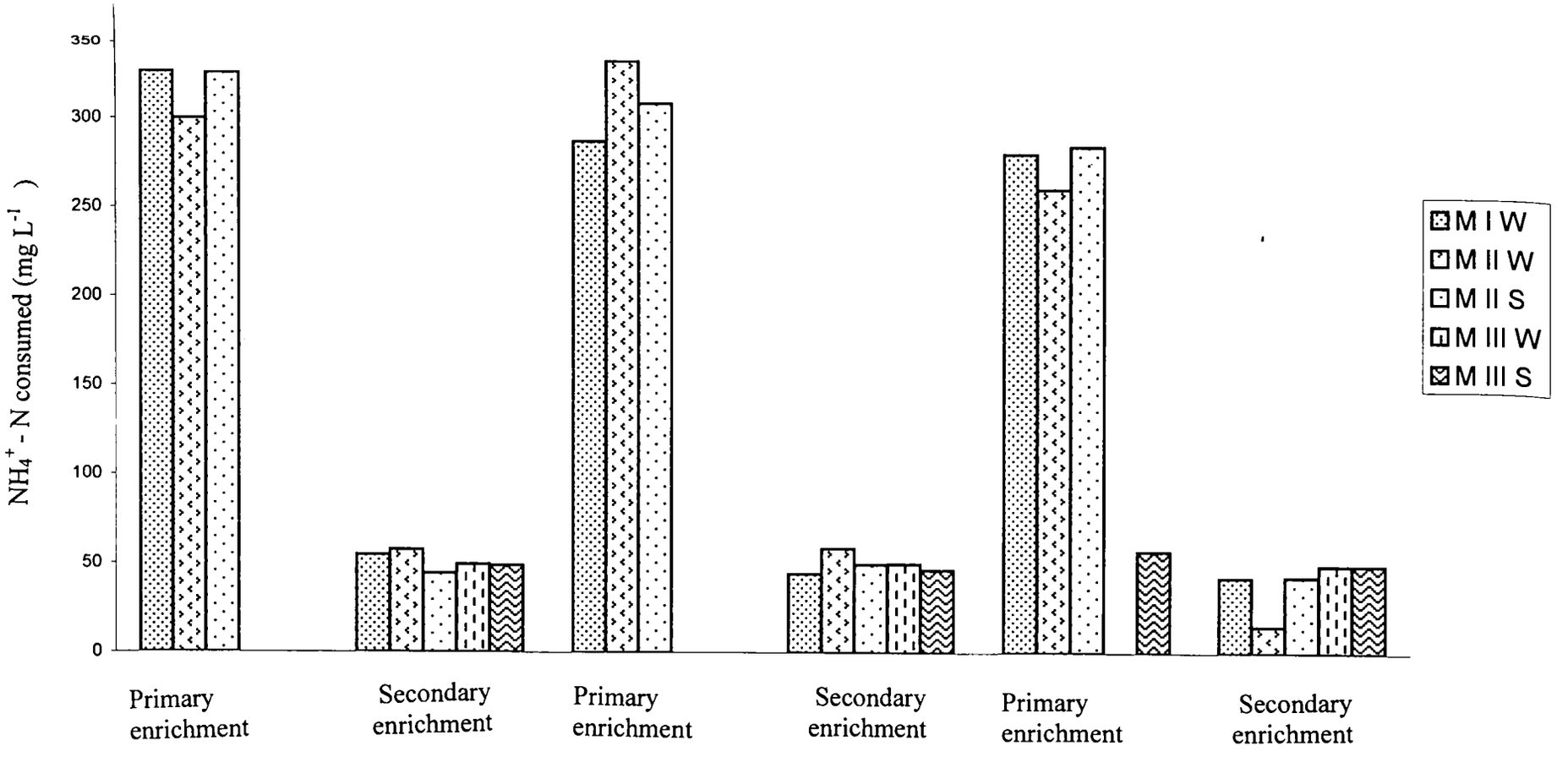


Fig 2.10 Comparison of the extent of growth attained by the consortia of ammonia oxidizers developed, during primary and secondary enrichment, for non-penaeid hatchery system

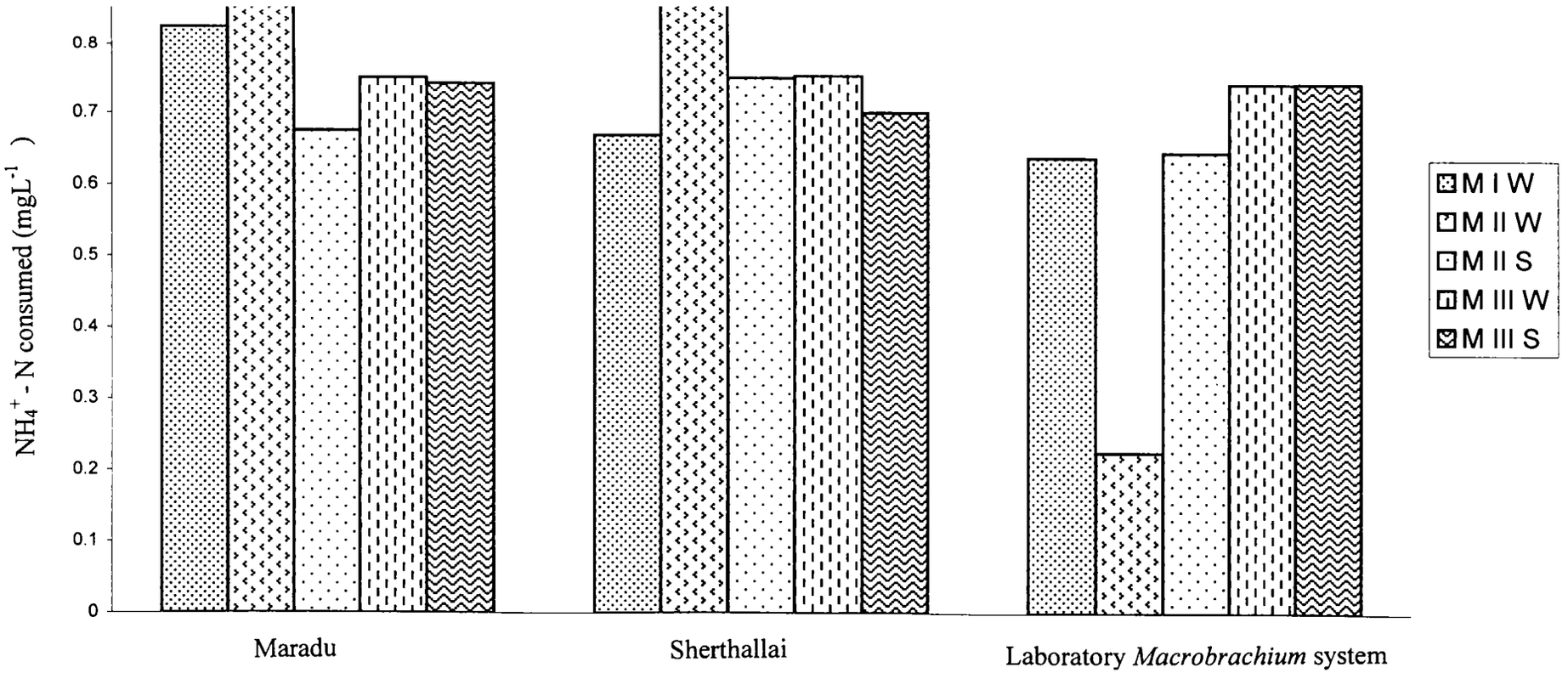


Fig 2.11 Comparison of the substrate consumed by the consortia of ammonia oxidizers over a period of 24 hours during secondary enrichment, for non-penaeid hatchery system

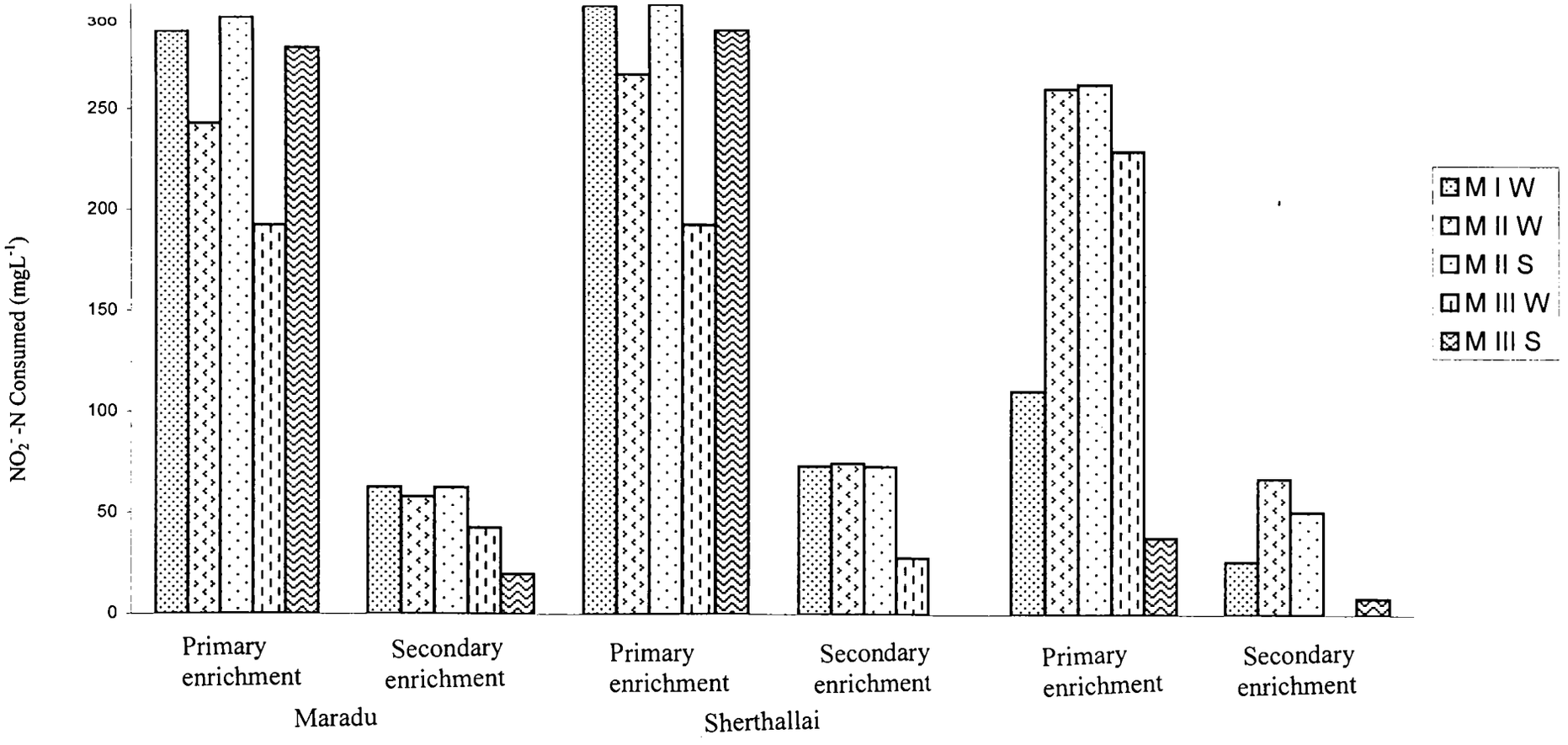


Fig 2.12 Comparison of the extent of growth attained by the consortia of nitrite oxidizers developed, during primary and secondary enrichment, for non-penaeid hatchery system

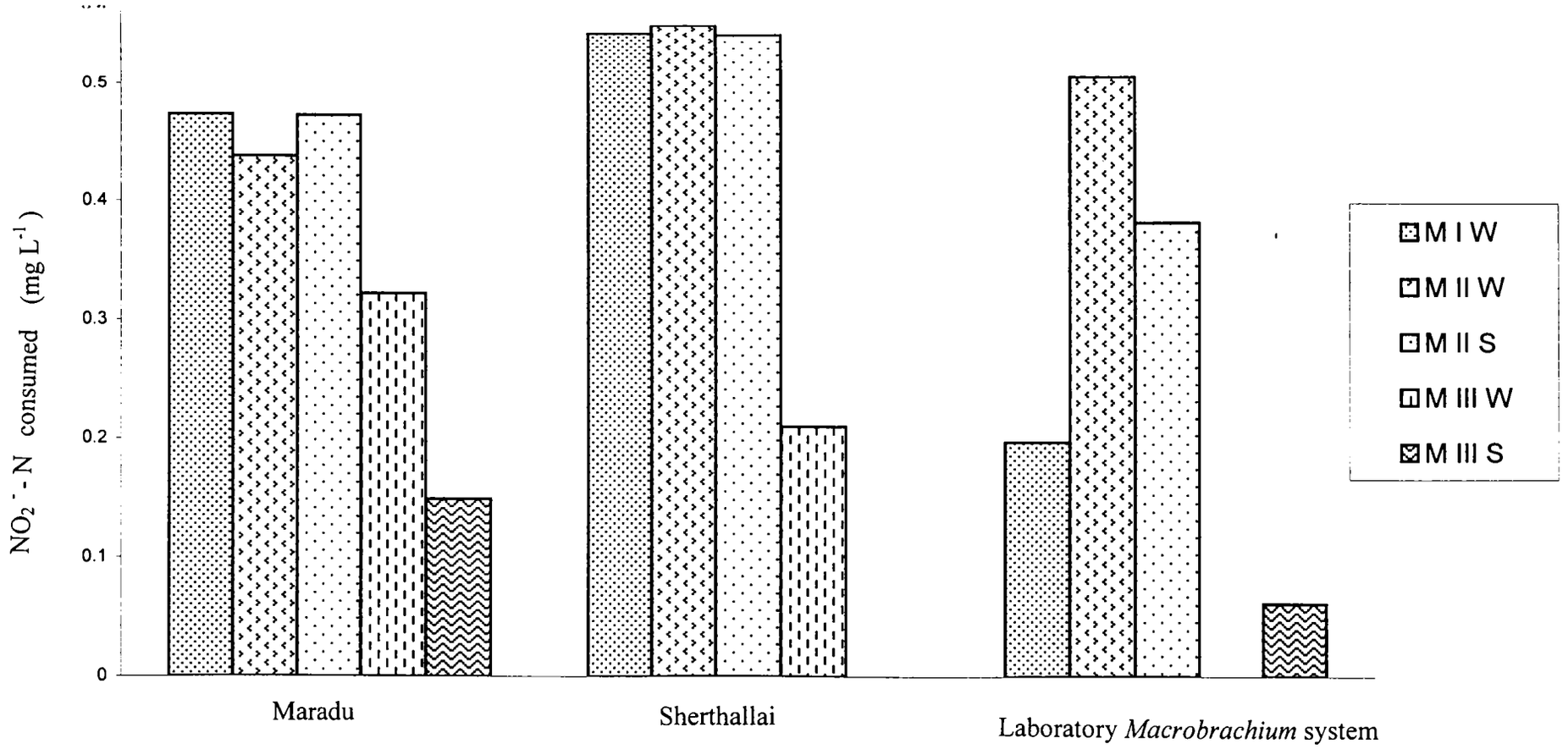


Fig 2.13 Comparison of the substrate consumed by the consortia of nitrite oxidizers over a period of 24 hours during secondary enrichment, for non-penaeid hatchery system

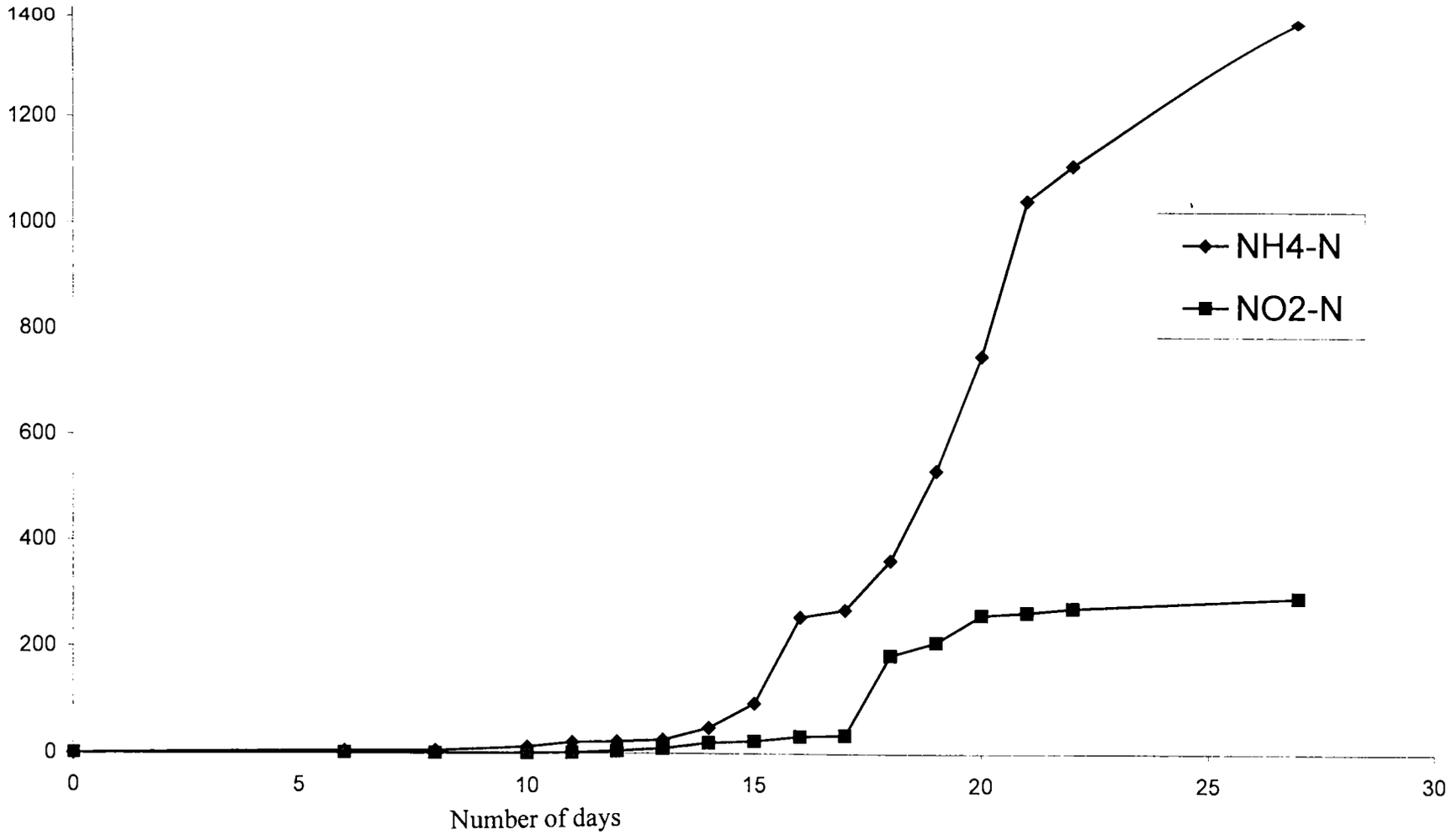


Fig 2.14 Growth pattern of AMOPCU I in fermentor

NH_4 - N : NH_4^+ - N consumed (mg L^{-1})

NO_2 - N : NO_2^- - N produced (mg L^{-1})

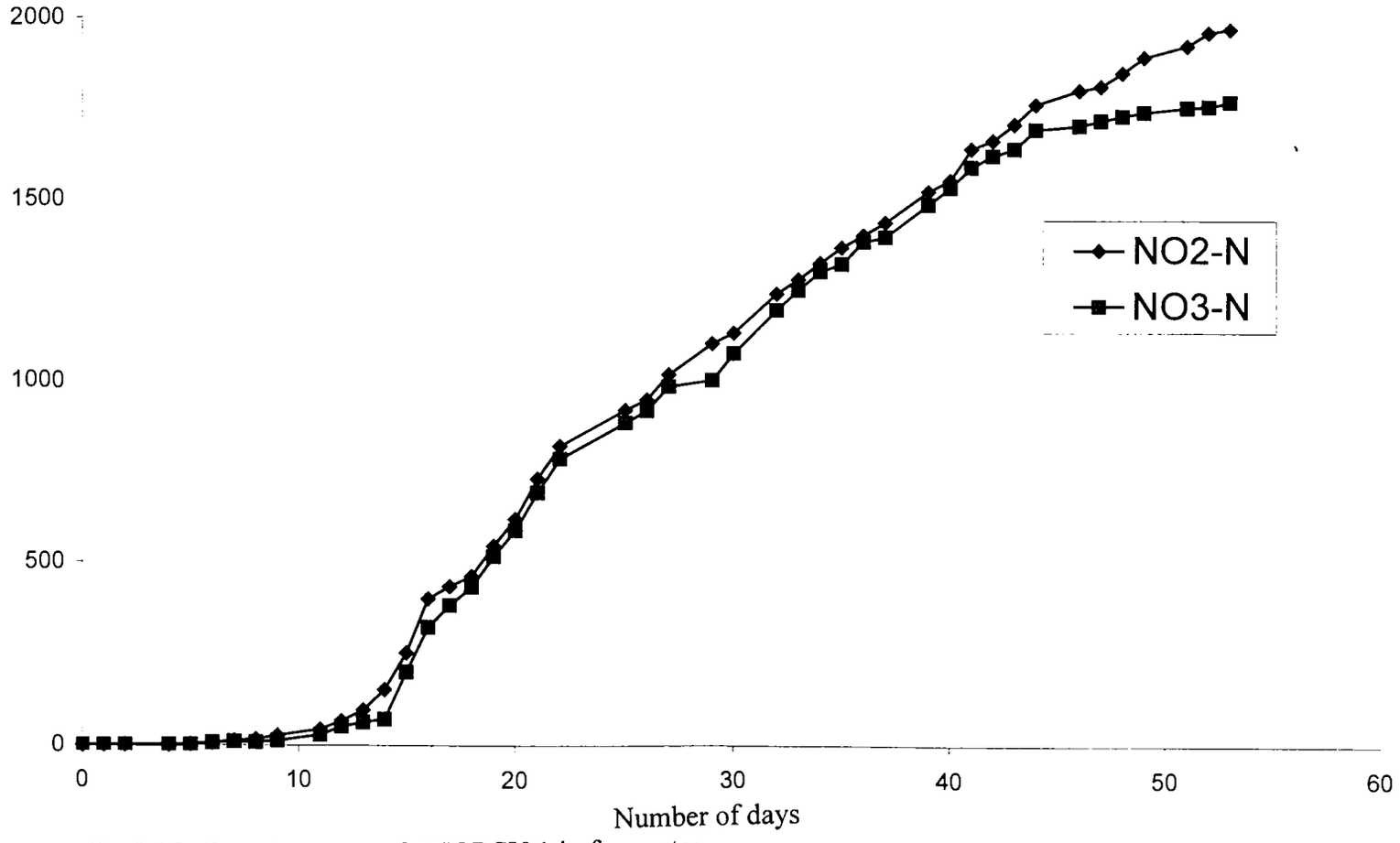


Fig 2.15 Growth pattern of NIOPCU 1 in fermentor

NO_2 - N : NO_2^- - N consumed (mg L^{-1})

NO_3 - N : NO_3 - N produced (mg L^{-1})

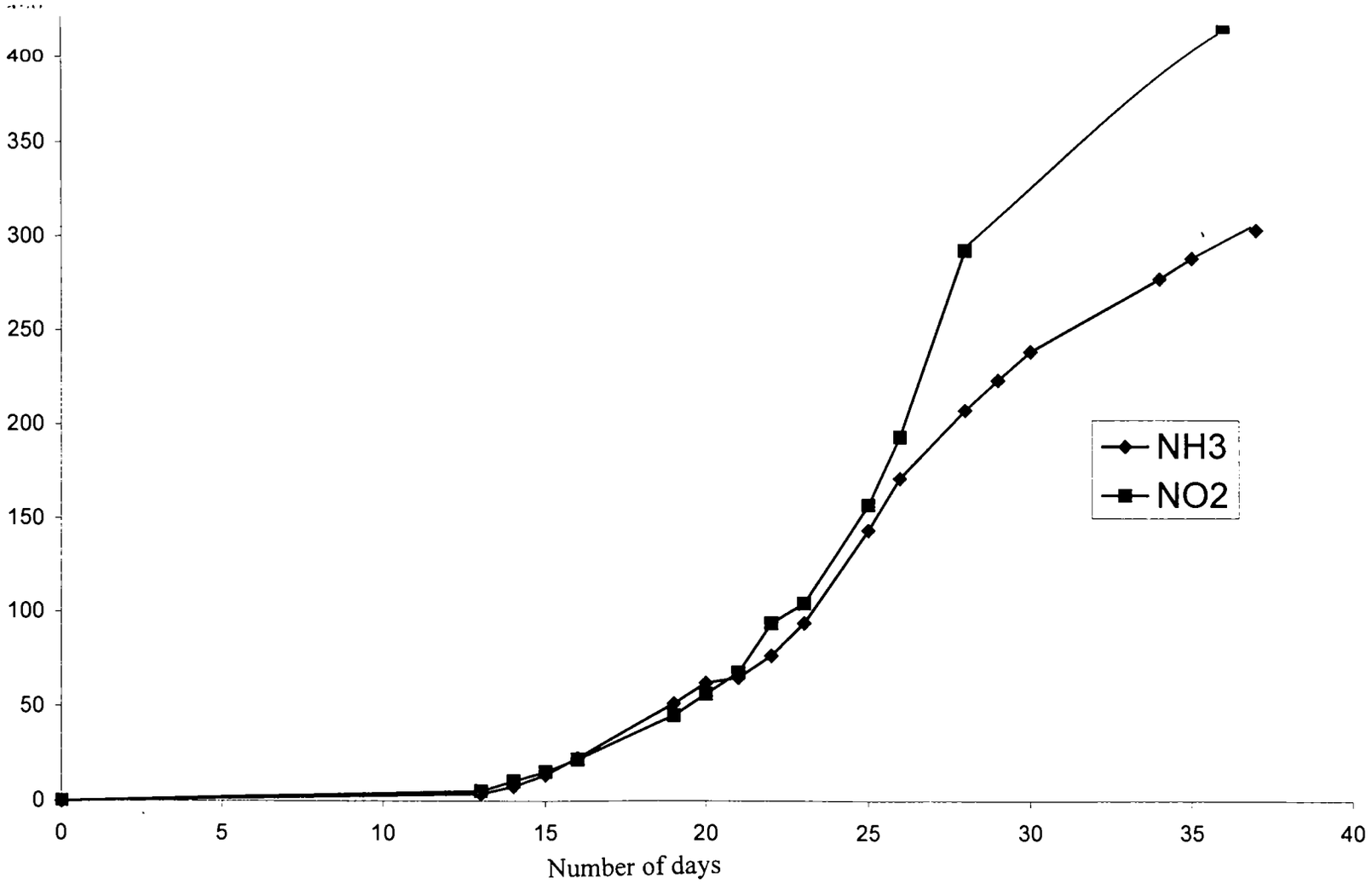


Fig 2.16 Growth pattern of AMONPCU 1 in fermentor

NH_4 - : NH_4^+ - N consumed (mg L^{-1})

NO_2 - : NO_2^- - N produced (mg L^{-1})

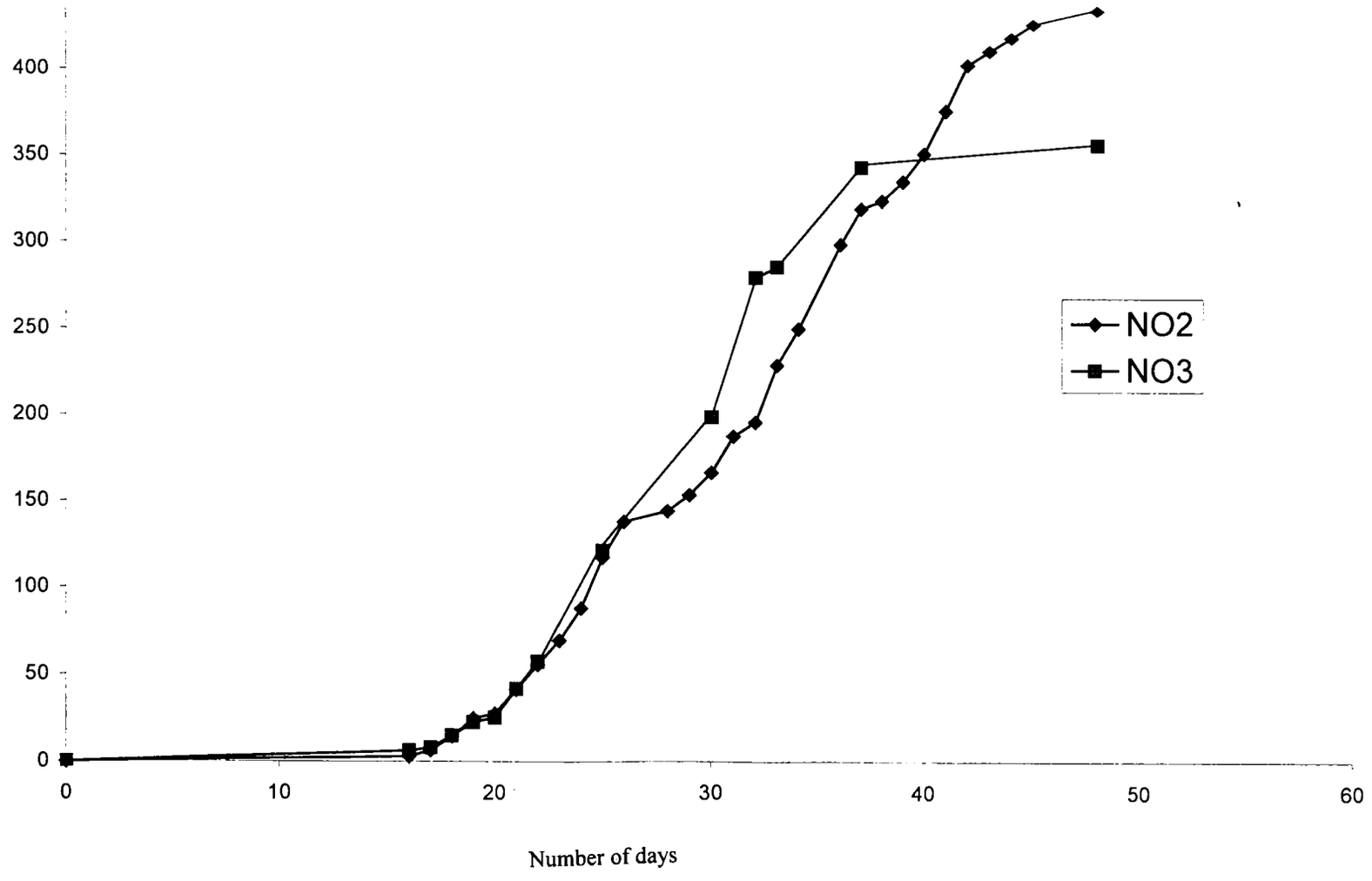
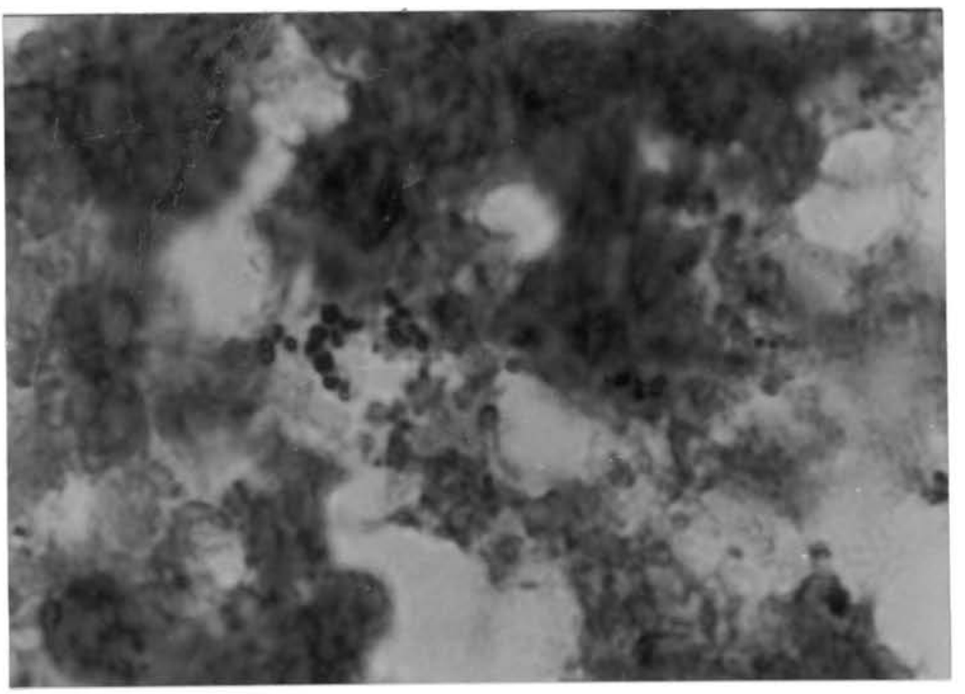


Fig 2.17 Growth pattern of NIONPCU I in fermentor

NO_2 : NO_2^- - N consumed (mg L^{-1})
NO_3 : NO_3 - N produced (mg L^{-1})

Fig 2.18 Gram stained cells of AMOPCU 1 (1000 X)

Fig 2.19 Gram stained cells of NIOPCU 1 (1000 X)



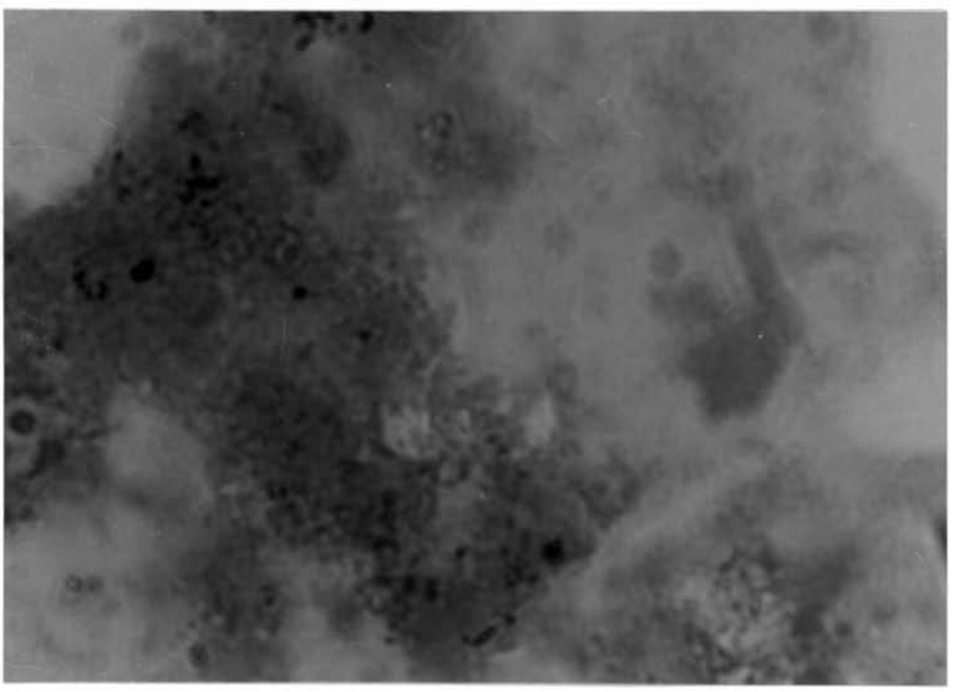
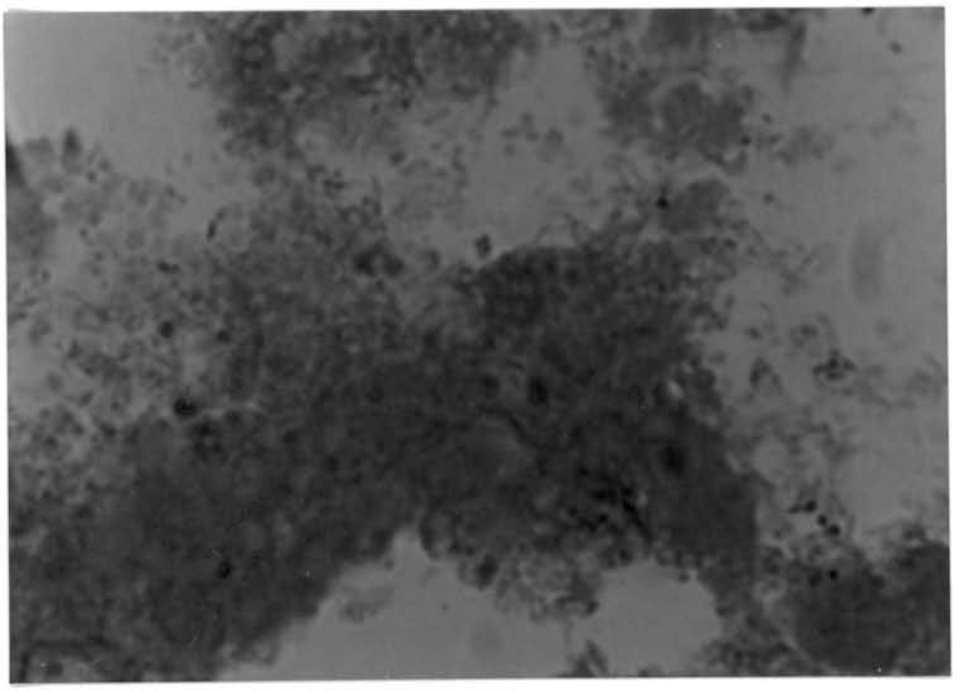
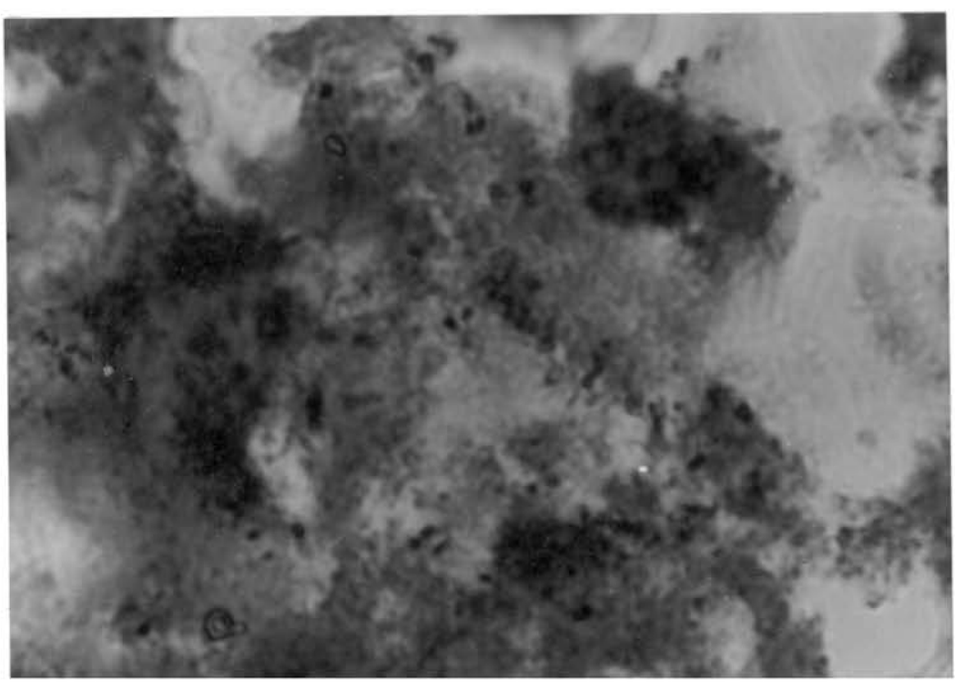


Fig 2.20 Gram stained cells of AMONPCU 1 (1000 X)

Fig 2.21 Gram stained cells of NIONPCU 1 (1000 X)





CHAPTER 3

MASS PRODUCTION AND CHARACTERIZATION OF NITRIFYING CONSORTIA

CHAPTER 3

MASS PRODUCTION AND CHARACTERIZATION OF NITRIFYING CONSORTIA

3.1 Introduction

As next step towards the development of nitrifying bioreactors the four nitrifying consortia such as AMOPCU 1, NIOPCU 1, AMONPCU 1 and NIONPCU 1 has to be mass produced so that sufficient nitrifying biomass would be available. Since the optimum growth requirements of these consortia have already been determined, mass production could be carried out under these conditions in a fermentor. While doing so, the total biomass which could be generated can be estimated along with other growth kinetic factors such as relationship between substrate uptake and product formed, total nitrifying cells, alkalinity-pH relationship, yield coefficient, generation time and specific growth rate. These growth kinetic factors are important when the whole mass production technology is scaled up for commercial production.

Compared with most heterotrophs, nitrifiers are slow growers. In liquid cultures, maximum specific growth rates for both groups of nitrifiers are in the order of 1.0 to 2.0 d^{-1}, the fastest reported being 2.2 d^{-1} with a doubling time of 8 hours (Skinner and Walker, 1961), with low cell yield (Prosser and Cox, 1982). It was considering these difficulties in getting sufficient biomass over a period of time that the optimum growth requirements has been worked out earlier. Accordingly the growth kinetics of the four nitrifying consortia were determined in a fermentor under optimum conditions.

Generally speaking, growth yield of nitrifying bacteria is very low and Skinner and Walker (1961), on experimenting with *N. europaea* obtained a yield of 0.71 g dry weight L^{-1} in a continuous culture. This is because of their

autotrophic nature and the low energy yields obtained from the oxidation of ammonia and nitrite. Because of the difficulty in obtaining sufficient cells, the biochemical studies on nitrifying bacteria have always been hindered (Prosser and Cox, 1982).

Even in conditions designated to favour the chemoautotrophic nitrifiers, contaminants build up rapidly and reach high population densities making the isolation of nitrifying bacteria in pure culture justifiably a difficult task (Clark and Schmidt, 1966). Even if pure cultures are obtained somehow or other, maintenance becomes very difficult and large scale cultures are susceptible to contamination by heterotrophs. In this context the observation made by Gundersen (1966) assumes paramount importance that the nitrification reaction is observed frequently to proceed more rapidly in the presence of heterotrophic microorganisms in mixed culture situation. Organic metabolites formed by the chemosynthetic bacteria could be accounted for the extensive development of heterotrophs in inorganic nitrification media. However, the benefits, which the nitrifiers obtain out of this relationship, are not known. The frequently suggested possibility that organic compounds formed by the heterotrophs may stimulate growth of associated autotrophic nitrifiers is without experimental support (Clark and Schmidt, 1966). As suggested by Jones and Paskins (1982) the heterotrophic bacteria totally depend on the catabolic products of nitrifying bacteria as the source of carbon as the media do not contain any available source of carbon. This realization led to maintain the consortium as such without being resolved and, purified and, the consortium was treated as a single unit for all practical purposes.

For the mass production of nitrifiers several people have tried different ways, Watson *et al.*, (1981) grew nitrifying bacteria in 20 litre semicontinous and continuous culture for ammonia and nitrite oxidizing consortium, and these methods yielded 0.5 g or more (wet weight) of cells daily. As mandatory these mass cultures were inoculated with at least one litre of a turbid culture. If heavy inoculum was not used the culture either did not grow or underwent a very long lag period.

The growth of nitrifying bacteria is usually measured by either substrate utilization or product formation. The benefits of measuring specific growth rate of nitrifying bacteria by using changes in substrate and product concentrations are thus enormous, where alternative measurements based on enumeration or determination of biomass are not possible in nitrifying consortia. Under conditions of balanced growth, for example, during exponential phase in batch culture, product concentration increases exponentially. When the stationary phase is reached these values get reduced drastically (Fliermans *et al.*, 1974, Fliermans and Schmidt, 1975).

One approach to the determinations of specific growth rate of the nitrifier component of a consortium involving heterotrophic organisms also is to estimate the yield co-efficient and the saturation constant. Another approach for estimating the concentration of nitrifiers in a mixed culture is by determining the specific activity of nitrifying bacteria as it is constant at a given temperature and pH. A specific activity co-efficient (α-k mixed culture/k pure culture) is determined for a mixed sample culture and a pure culture of nitrifiers. It is argued that α equals the fraction of mixed culture TKN that is contributed by the nitrifiers. The concentration of nitrifying bacteria is estimated by multiplying α by TKN L^{-1} of the mixed culture. This approach has several drawbacks. It neglects substrate utilization for exogenous respiration or maintenance. The estimated generation time for *Nitrosomonas* sp. is in the range of 8-36 hours and for *Nitrobacter* sp. in the range of 12-59 hours (Sharma and Ahlert, 1977). It has been demonstrated that (Belser and Schmidt, 1980; Keen and Prosser, 1987a) that product concentration increases exponentially during batch growth of nitrifiers and Hoffman and Lees (1952) suggested that the product accumulation will lead to decrease in efficiency.

When the growth of a batch culture is followed by means of dry weight determination, the growth parameters of primary interest are the cell yield (Y), the

exponential growth rate and the duration of lag phase. Sharma and Ahlert (1977) obtained cell yield 'Y' for ammonia oxidizers as 0.03-0.13 and 'Y' for nitrite oxidizers lies in the range 0.02-0.08. Estimated values of 'Y' from theoretical consideration on the thermodynamics of growth are 0.29 for ammonia oxidation and 0.084 for nitrite oxidation.

As far as the storage and maintenance of nitrifying bacteria are concerned the first choice goes to liquid nitrogen where the nitrifiers can have a greater than 90% survival rate for an indefinite time period (Watson *et al.*, 1981). As cryoprotectant, dimethyl sulphoxide (DMSO) is added to an actively growing culture to a final concentration of 5 %. An aliquot of the culture is then sealed in a 2 mL ampule, cooled at a rate of 4^0C per minute in an acetone dry ice bath to -20^0C and then stored in a liquid nitrogen cryostat. For activating the cryopreserved culture Watson *et al.*, (1981) thawed the ampule at 37^0C and the culture was serially diluted so that the DMSO concentration was diluted to a non-inhibitory level for growth. By this way any contaminants introduced during the freezing procedure were diluted out.

For short (4-6 months) maintenance, liquid stock cultures can be stored at 15^0C and transferred to fresh media at the end of the period (Watson et al, 1981). The concentration of substrates in the culture should be analyzed periodically and increments added when needed. Prior to subculturing the stored culture should be incubated at 25^0C for several days.

In literature, attempts to freeze dry nitrifying bacteria are seldom seen. This is mainly due to the difficulties in obtaining sufficient quantities of biomass. Instead cultures are usually stored in liquid medium in sealed containers which are either refrigerated (Prosser, 1982), and subcultured every 4-6 weeks.

One of the questions asked was, could the cultures be centrifuged and the cell pellet be resuspended in fresh medium without loss of activity. It is highly

advantageous if we can reduce the volume of the culture without the loss of activity. One of the basic aims of maintenance and preservation of bacteria are to keep cultures viable, uncontaminated and unchanged in their properties. For very long-term preservation, involving stocks of the strains and where withdrawals from stocks are regularly made, a fourth aim is to have adequate stocks and appropriate systems for replenishing the stocks when necessary. In the present context the consortia generated in the fermentor have to be used in the reactors. Storage of the cultures is a problem that has to be addressed at commercial level when large volumes of cultures will have to be maintained.

Generally, the ammonia oxidizing bacteria are categorized taxonomically by their shape, size and arrangement of membranes within their cytoplasm. In all such studies, pure culture have been used and such cells have been observed to be rod shaped spherical, spirullar or lobular without endospores. Some but not all, species possess intracytoplasmic membranes, which may occur as flattened lamellae arranged internally, peripherally or randomly. Most cells have a typical gram negative cell envelope. When cells are motile, flagellae are polar to lateral or peritrichous. In enrichment culture and in nature, ammonia oxidizing bacteria frequently occur in cell aggregates, reformed to as zoogloea or cysts. A zoogloea contain loosely associated cells embedded in a soft slime layer, while a cyst contains closely packed and compressed cells firmly embedded in and surrounded by a tough slime layer (Watson, *et al.*, 1981). Ramachandran (1998) on studying with two consortia of nitrifying bacteria observed the cells aggregated and covered with a less electron dense slime secreted by the organism. Through these slimy mass, cells could make interconnections and remained entangled.

3.2 Materials and Methods

3.2.1 Mass culture of ammonia oxidizing consortia for penaeid hatchery (AMOPCU 1)

The medium according to Watson (1965), prepared in 30 ppt seawater was used for mass culture of ammonia oxidizing consortia meant for penaeid hatchery. Fermentor (New Brunswick BIOFLO 2000) vessel was autoclaved at 10 lbs. for 10 minutes with medium in it. After autoclaving, fermentor vessel was reassembled with control panel and appropriate growth conditions such as pH 7.5, temperature 28^0C, salinity 30 ppt and substrate concentration of 10 $\mu g\ mL^{-1}$ was provided. A 1% inoculum (v/v) was given and the agitation was set at 200 rpm with an airflow rate of 0.6 L min^{-1}. Entry of light was prevented by covering the fermentor vessel with black cloth. Once in 24 hours, NH_4^+-N consumed and NO_2^--N, produced were noted. As and when pH drop was noticed, it was automatically adjusted by addition of 10% Na_2CO_3 from the base port. As the consumption of NH_4^+-N progressed, it was supplemented with fresh aliquots of NH_4^+-N, at an exponential rate and 10 $\mu g\ mL^{-1}$ at a time. After attaining the stationary phase characterized by decline in the production rate of NO_2^--N, the culture was harvested fully after dislodging the wall growth and was subsequently maintained in screw capped bottles at 4^0C in a refrigerator.

3.2.2 Mass culture of nitrite oxidizing consortium for penaeid hatchery (NIOPCU 1)

Procedure followed for the mass culture of NIOPCU 1 was the same as that of AMOPCU 1. From the amplified consortia which was centrifuged and stocked at 4^0C, a 1 % inoculum was supplied. Optimum conditions experimentally noted for this culture like pH 7, temperature 37^0C, and salinity 25 ppt and NO_2^--N concentration of 10 $\mu g\ mL^{-1}$ were provided. Agitation of 200 rpm with an airflow rate of 0.6 L min^{-1} was provided and fermentor vessel was covered with black

cloth. Once in 24 hours, NO_2^--N consumption and NO_3-N production was quantified. As the consumption of NO_2^--N progressed, the medium was supplemented with fresh aliquots of NO_2^--N not exceeding at a time 10 $\mu g\ mL^{-1}$, at an exponential rate. After attaining stationary phase, in terms of NO_2^--N consumption and NO_3-N, the wall growth was dislodged to the maximum possible extent and the culture was harvested. It was maintained in screw capped glass bottles at 4^0C. Since there were no pH drop, the need to adjust pH did not arise.

3.2.3 Mass culture of ammonia oxidizing consortium for non-penaeid hatchery system (AMONPCU 1)

In the case of ammonia oxidizing consortia for non-penaeid hatchery system, the optimum requirements were pH 8.5, temperature 28 ^{0}C and salinity 10 ppt and substrate concentration of 10 $\mu g\ mL^{-1}$. In sterile fermentor vessel with sterile media, a one per cent inoculum from previously amplified stock culture was given. Optimum requirements were provided, with 200 rpm agitation, an air flow rate of 0.6 L min^{-1} and maintained in dark. NH_4^+-N and NO_2^--N concentration was noted every 24 hours to assess the growth of the culture and exponential addition of NH_4^+-N was given not exceeding 10 $\mu g\ mL^{-1}$ at a time. As the culture attained stationary phase, indicated by reduction in substrate consumption and product formation, harvesting was done. During growth phase, as and when pH drop occurred, it was adjusted using 10% Na_2CO_3 automatically. The harvested culture was stocked at 4^0C, in screw capped bottles.

3.2.4 Mass culture of nitrite oxidizing consortium for non-penaeid hatchery system (NIONPCU 1)

The fermentor vessel was autoclaved along with media prepared in 5 ppt seawater which is the optimum salinity noted. Eventhough optimum pH noted experimentally was 4, it was retained at 7, because it was not practical to use a

culture, which was grown at pH 4, in a hatchery system which requires almost neutral to alkaline pH. Moreover, throughout the period of enrichment this culture had been at pH of 7.5. Therefore it was assumed that eventhough the optimum pH requirement was recorded as 4, the culture might grow well at pH 7 as well. Optimum temperature of 45^0C and initial substrate concentration of 10 $\mu g\ mL^{-1}$ with agitation rate of 200 rpm and an air flow rate of 0.6 L min^{-1} was given and the vessel was covered with black cloth to prevent the entry of light. NO_2^--N and NO_3-N was assessed daily. Since even after a period of one month with no sign of growth being observed, the experiment, was forced to be terminated.

The whole experiment was repeated once more and since no growth was observed even after 32 days of incubation this was also terminated. Since there was an absolute need for a culture which would function at 15 ppt salinity, it was decided to acclimatize the nitrite oxidizer enriched from the 30 ppt penaeid hatchery (NIOPCU 1) to 15 ppt and employ it in the reactor. During the experiment executed to find the optimum salinity it was observed that the 30 ppt nitrite oxidizing consortia (NIOPCU 1) could grow well at 15 ppt also. From the mass cultured and stocked culture of NIOPCU 1, 250 mL was transferred into a 1 L conical flask and kept over a magnetic stirrer. Daily the activity was monitored in terms of NO_2^--N consumption and NO_3-N production. The salinity was gradually reduced from 30 ppt to 15 ppt by addition of media prepared in distilled water at a rate of 1 unit per day after ensuring that nitrification was not inhibited. When the salinity reached 15 ppt, the culture was allowed to stabilize for 2-3 days. Thereafter the fermentor was set with sterile medium having pH 7.0, temperature 37 ^{0}C, substrate (NO_2^--N) concentration of 10 $\mu g\ mL^{-1}$, and agitation of 200 rpm, airflow rate of 0.6 L min^{-1} and the vessel was covered with black cloth. Growth in terms of NO_2^--N consumption and NO_3-N production was noted every 24 hours. As and when substrate consumption was noted, it was replenished with fresh aliquots of NO_2^-. N, which was, added exponentially but not exceeding 10 $\mu g\ mL^{-1}$ in a single addition. On attaining stationary phase, the

culture was harvested fully and stocked in screw capped glass bottles at 4^0C. This culture will be henceforth called as NIOPCU 1a.

3.2.5 Determination of biomass

When the stationary phase was attained, the entire culture was drained after dislodging the wall growth. To determine the total biomass 1 mL each of the culture from all the four cultures (AMOPCU 1, AMONPCU 1, NIOPCU 1, and NIONPCU 1a modified NIOPCU 1 to suit non-penaeid system) were filtered through dried, preweighed membrane filter disc (Millipore Pvt. Ltd. Bangalore) having pore size of 0.22 μm. The filter discs were then dried in hot air oven set at 80^0C and subsequently in a desicator till constant weight was obtained. The difference in weight gave the dry weight of biomass generated per mL. From this, biomass per 2000 mL was computed.

3.2.6 Relationship between substrate uptake and product formed

The relationship between percentage consumption of NH_4^+-N and build up of NO_2^--N of the consortia AMOPCU 1 and AMONPCU 1 and percentage consumption of NO_2^--N and build up of NO_3-N of the consortia NIOPCU 1 and NIONPCU 1a were worked out from the overall consumption of substrate and build up of products under optimum growth conditions in the fermentor.

3.2.7 Enumeration of the consortia developed

Ammonia oxidizing and nitrite oxidizing consortia developed were enumerated based on the following relationship (Belser, 1974; Ardakani, 1974a; Schmidt, 1974; Voltz *et al.*, 1975a,b)

Ammonia oxidizers : $3x10^4$-$1.2x10^5$ cells/μg NH_4^+-N oxidized

Nitrite oxidizers : 1-$4x10^4$ cells/μg NO_2^--N oxidized

3.2.8. Alkalinity-pH relationship

During ammonia oxidation, due to hydroxylamine production, pH drops to acidic ranges and it is adjusted to optimum level using 10 % sodium carbonate. The alkalinity destroyed due to growth of ammonia oxidizers is calculated based on the relationship, 6.0-7.4 mg alkalinity is destroyed per milligram NH_4^+-N oxidized to nitrite (EPA-1975)

3.2.9. Determination of yield coefficient or cell yield 'Y' of the consortia

Yield coefficient can be defined as the weight of cells generated to the weight of substrate oxidized. It is also the ratio of weight of product formed to the weight of substrate utilized (Sharma and Ahlert, 1977)

$$\text{Yield coefficient } Y = \frac{\text{Weight of cells generated}}{\text{Weight of substrate oxidized}}$$

[Or]

$$\frac{\text{Weight of product formed}}{\text{Weight of substrate utilized}}$$

Based on substrate oxidized, product formed and biomass generated, yield coefficient for all the cultures were calculated.

3.2.10. Determination of generation time (tg)

Generation time or doubling time for a culture is the time taken for the biomass to double its initial amount, or the product build up to double the rate.

For ammonia oxidizers the generation time was calculated based on the time taken for doubling the NO_2^--N build up and for nitrite oxidizers, based on the time taken for doubling the NO_2^--N consumption.

3.2.11. Specific growth rate (μ)

Specific growth rate is inversely proportional to the generation time i.e. the product of generation (tg) and specific growth rate is a constant.

i.e., μ tg = 0.693

or μ = 0.693/tg

The specific growth rate μ of ammonia and nitrite oxidizing consortia were calculated from their generation time.

3.2.12 Microscopy of nitrifying consortia

3.2.12.a Phase contrast microscopy

For observing under the phase contrast microscope (Ophiphot.Japan), small aliquots of consortia were removed from refrigeration and a loopful of which was placed on microscope slide, put a cover slip and observed under oil immersion objective of phase contrast microscope (Ophiphot. Japan).

3.2.12.b Scanning electronmicroscopy.

An aliquot of 1ml each consortium was centrifuged at 10,000 rpm in a refrigerated centrifuge (Remi, Pvt Ltd) for 15 minutes. The pellet was fixed in 2.5% glutaraldehyde prepared in seawater with respective salinity (30 ppt for AMOPCU 1 and NIOPCU 1 and 15 ppt for AMONPCU 1 and NIONPCU 1a) at 4°C for 24 hours. The vials were centrifuged and washed with seawater of respective salinity and the pellet was post fixed in 2% OsO_4 prepared in seawater

with the same salinity. The bacterial suspension was repeatedly washed in sea water having respective salinity and dehydrated through an acetone series of 70 to 100%.

For SEM cells were first adsorbed on to cover glass surface, dehydrated through an acetone gradient and critical point drying is done using CO_2 as the transitional fluid. The cover glasses were then mounted with colloidal silver on aluminum stub and coated with gold on a sputter coater and examined.

3.3 RESULTS AND DISCUSSION

3.3.1 Substrate consumption and product build up

Pattern of substrate consumption and product build up of all the four consortia such as AMOPCU 1, NIOPCU 1, AMONPCU 1 and NIONPCU 1a under optimum growth conditions are summarised in Tables 3.1 to 3.4 and Figs 3.1. to 3.4. On consuming 703.79 g L^{-1} NH_4^+-N, the ammonia oxidizing consortium AMOPCU 1 could produce 611.31 g L^{-1} NO_2^--N which formed 86.86 % of the substrate utilized over a period of 27 days. After 21 days of culture as shown in Fig 3.1 there recorded a progressive decline in the rate of production of NO_2^--N compared to the NH_4^+-N consumed indicating its entry into a stationary phase. Meanwhile the nitrite oxidizing consortium NIOPCU 1 consumed 5157.59 mg L^{-1} NO_2^--N over a period of 49 days transforming 89.19 % to NO_3-N (4600.13 mg L^{-1}), by the time the culture had entered into stationary phase indicated by a lowering in the rate of nitrification (Fig 3.2). Contrary to the above observations the ammonia oxidizing consortium AMONPCU 1 on consuming 238.54 mg L^{-1} NH_4^+-N exhibited an output of 379.96 mg L^{-1} NO_2^--N. This interesting behaviour of the consortium was noticed from the 21st day of incubation (Fig. 3.3). This apparently indicated that the consortium started utilizing another source of NH_4^+-N for the build up of NO_3-N and consequently for deriving energy after 21 days of incubation in the fermentor. Behavior of this consortium could be

observed over and again in several occasions as documented in the succeeding pages. Same is the case with NIONPCU I a which consumed 2313.96 mgL^{-1} NO_2^--N and formed 2327.518 mgL^{-1} NO_3-N over a period of 18 days (Fig 3.4)

During the mass production of nitrifying consortium in the fermentor the culturing started with 10 $\mu g\ mL^{-1}$ substrate which was later increased exponentially as the consortium started consuming it. This was continued till signs of stationary phase was seen. In the case of stationary phase AMOPCU 1 and NIOPCU 1 87% and 89% of oxidation of NH_4^+-N and NO_2^--N were oxidized to the end products of nitrification. This is contrary to the observation made by Ramachandran (1998) where the products of nitrification of two nitrifying consortia developed from sewage was 19.39 % to 19.88 % of the substrate consumed. This indicates the quality of nitrifying consortia developed here in terms of the lesser proportion of the heterotrophs contained in them. On the contrary the consortium AMONPCU 1 and NIOPCU I a performed in such a way that the product output was higher than the substrate consumption suggesting the possibility of an extraneous source of NH_4^+-N for nitrification.

During the period of culture, biomass generated could not be determined periodically, as there was tremendous wall growth. Finally on termination of the experiment the consortia were scrapped off the fermentor vessel and the biomass assessed gravimetrically.

The study strongly suggest that for getting good quality consortium, i.e., consortium with lesser heterotrophs and more than 90% nitrifiers, the culturing should be terminated when the rate of building up of the products of nitrification such as NO_2^--N in the case of ammonia oxidizers and NO_3-N in the case of nitrite oxidizers begins to slacken, during which the consumption of substrate will still be going on unhampered. This indicates that the nitrifiers have entered into stationary phase while the associated heterotrophs are not. But if we continue culturing, the biomass build up may go up but that would be more of heterotrophs

than of nitrifiers. According to Prosser (1989), pure cultures of *Nitrosomonas* and *Nitrobacter* used up 76 % of NH_4^+-N and 81 % of NO_2^--N for maintenance and not for cell yield. In the case of the four consortia developed here greater than 85 % substrate (Table 3.5) could be converted to the end products indicating that the energy released was utilized for the fixation of CO_2. However the biomass which could be assessed at the end of the culturing period was very low and the basic reason for this low biomass yield is undoubtedly the wall growth and the difficulty encountered in dislodging it from the fermentor. Therefore on commercializing the technology an appropriate method has to be evolved for the safe dislodgment of cells without loosing their viability and nitrifying potential.

3.3.2 Enumeration of nitrifying bacteria in the consortia developed

Nitrifying bacteria in the consortia developed were enumerated and summarized in Table 3.6. Nitrifiers in the ammonia oxidizing consortia (AMOPCU1 and AMONPCU 1) ranged from 1.833×10^{13} - 7.33×10^{13} to 4.56×10^{13} – 18.4×10^{13} cells L^{-1}. Meanwhile nitrifiers in NIOPCU I and NIONPCU I ranged from 4.6 to 18.4×10^{13} cells L^{-1} and 2.33×10^{13} This clearly indicated that between consortia, difference in the cell number of nitrifiers was quite marginal, affirming the suitability of the culture conditions provided in the fermentor. It has to be emphasized that all growth conditions provided were optimum, which was determined earlier for each consortium. The data further suggests that all consortia were harvested at the right moment, before the augmented growth of heterotrophs which might happen at the expense of dying nitrifiers. According to Alexander (1965) *Nitrosomonas* oxidizes about 35 atoms of nitrogen and *Nitrobacter* oxidizes 100 atoms of nitrogen (2.86 times more) for the fixation of a molecule of CO_2. Even though this happens to be the stoichiometry of nitrification, in the present context the substrate required for nitrite oxidizers is almost 10 times higher to that required by ammonia oxidizers. In other terms, to support almost the same biomass by nitrite oxidizers, 10 times more of NO_2^--N had to be oxidized. Results almost comparable to this have been

observed by Belser (1974); Ardakani *et al.*, (1974 a) and Schmidt (1974), who observed that about 1 to 4 x 10^4 cells of *Nitrobacter* per kg N are produced from the oxidation of nitrite. Volz *et al.*, (1975 a and b), observed that the same quantity of ammonia nitrogen could support almost three times the cells of *Nitrosomonas* in soil with a continuous perfusion of ammonia. The marginal difference in the cell density between the four consortia developed here implies that the stationary phase is related to both the cell number of nitrifiers and the product accumulated in the fermentor.

3.3.3 Alkalinity – pH relationship

To compensate the lowering of pH and to bring it back to optimum during the growth of ammonia oxidizing consortia such as AMOPCU 1 and AMONPCU 1 in fermentor, the quantity of 10 % sodium carbonate required was estimated and the total alkalinity destroyed are summarized in Table 3.7. In the case of the consortium AMOPCU 1 where 703.79 g L^{-1} NH_4^+-N was removed, the total alkalinity destroyed was 4222.74 –5193.47 and to compensate that 188 mL 10 % sodium carbonate was added to the 2L fermentor. As far as the ammonia-oxidizing consortium AMONPCU 1 was concerned the total NH_4^+-N removed, was 238.54 g L^{-1} during which the alkalinity destroyed was 1431.21-1765.16 and to compensate this 105 mL 10 % sodium carbonate had to be added to the 2L fermentor. The values indicate comparatively better performance of the cultures leading to a higher biomass output. In a similar study conducted by Ramachandran (1998) an ammonia oxidizing consortium developed from sewage on growing in a fermentor under optimum conditions destroyed only 25.74 -31.74 g alkalinity per litre during the period of culture. This information is of high practical value as the sodium carbonate required adds to the cost of production of nitrifiers. For every gram NH_4^+-N oxidized by AMOPCU 1, 0.27 mL 10 % sodium carbonate was required and is same in the case of AMONPCU 1 it was 0.44 mL alkali. This variation is linked with the optimum pH of the consortia as the optimum of AMOPCU 1 was 7.5 and that of AMONPCU 1 was 8.5.

3.3.4 Yield coefficient

The yield coefficient 'y' of the ammonia and nitrite oxidizing consortia were determined and summarized in Table 3.8. The yield coefficient of AMOPCU 1 ranged from 0.109 to 0.869 and that of AMONPCU 1 ranged from 1.26 to 1.59 indicating that both the consortia differ very much in their potential to nitrify. The consortium AMONPCU 1 is found to be more efficient. For this culture the yield coefficient when calculated in both the ways, identical values were obtained which apparently indicated that the biomass generated could be recovered fairly well at the end of the culturing period. Meanwhile in the case of AMOPCU 1 the yield coefficient calculated based on the cell yield was around eight times lesser than that obtained on the calculation based on the generation of NO_2^- -N which indicated tremendous wall growth and loss of biomass during harvesting. In the same way the nitrite oxidizing consortia, NIOPCU 1 and NIONPCU 1a showed very low yield coefficient on calculating them based on the cell yield indicating the loss of biomass during harvesting. But the estimated value of 'y' from theoretical consideration on the thermodynamics of growth are 0.2 for ammonia oxidizers and 0.084 for nitrite oxidizers. In the same way Sharma and Ahlert (1977), obtained yield coefficient 'y' for ammonia oxidizers as 0.03-0.13 and for nitrite oxidizers as 0.02 to 0.08. Compared to these values the yield coefficient obtained here for both ammonia and nitrite oxidizers are very much higher indicative of the better energy conversion efficiency and higher carbon dioxide fixation potential of the consortia as NH_4^+-N and NO_2^- -N are the sole source of energy supplied in the medium. In almost similar way Ramachandran (1998) on experimenting with nitrifying consortia generated from sewage observed a comparatively higher value of 0.1864 to 0.1939 for ammonia oxidizers and 0.1745 to 0.1988 for nitrite oxidizers. This precisely indicates that the yield coefficient of nitrifiers from the tropics is higher compared to that from temperate regions. Besides this can also be due to the fact that heterotrophs are also associated with the chemolithotrophs as integral part of the consortium.

These heterotrophs live on the exudates /metabolites of chemolithotrophs as 'scavengers' and use them up as carbon and energy and probably as nitrogen source also. This 'secondary process' adds to the total biomass generated and reasonably justifies the comparatively higher yield co-efficient obtained. This may be considered a reflection of the efficiency of a consortium than pure cultures as far as the energy assimilatory capability is concerned (Ramachandran, 1998).

3.3.5 Generation time

Generation time or doubling time is the time required for the biomass to double its initial amount, or the time require to double the substrate consumption or the time to double the product build up in the present context. Here generation time for ammonia-oxidizing consortia was calculated based on the time required for doubling NO_2^- -N production, for nitrite oxidizers it was based on the time required for doubling NO_2^- -N consumption. This selection was made on the easiness and precision in the determination of NO_2^- -N than NH_4^+-N and NO_3-N or even the determination of biomass.

The generation time of the ammonia oxidizing consortium (AMOPCU 1) determined from the pattern of generation of NO_2^- -N in fermentor over a period of 27 days is summarized in Table 3.9 extrapolated from the Fig.3.5. The generation time was the lowest (10.50 hrs) in the initial phase of growth during which the product of nitrification accumulated was very little at 3.78 mg L^{-1}. Subsequently for every stage of doubling of the product the time required exhibited progressive protraction indicating that the culture was under severe inhibition which is normally designated as the product inhibition. The terminal stage of nitrification is marked by the prolongation of generation time to 175.2 hours indicating the entry into stationary phase.

The generation time of nitrite oxidizing consortium (NIOPCU 1) determined from the pattern of consumption of NO_2^--N in fermentor over a period of 49 days is summarized in Table 3.10 extrapolated from the Fig 3.6 . The shortest generation time of 22.34 hours was noticed during the initial phase of growth only. As the incubation progressed the duration required for doubling the consumption of NO_2^--N was found to increase almost exponentially. Naturally this prolongation in the generation time is due to the accumulation of NO_3-N, the product of nitrification.

Generation time of ammonia oxidizing consortium AMONPCU 1 determined from the pattern of generation of NO_2^--N in fermentor over a period of 29 days is summarized in Table 3.11 extrapolated from the Fig. 3.7. In the initial phase of the growth cycle it took 269 hours to produce 1.2 g L^{-1} NO_2^--N from NH_4^+-N. As the culture became very active, the duration for doubling the product NO_2^--N came down to 21 hours which subsequently increased to 82 hours at the stage of termination of experiment. In this case also product inhibition can be sited as the reason for the prolongation of generation time in the final stage.

The generation time of NIONPCU I a (NIOPCU I acclimatized to 15 ppt salinity) over a period of 47 days based on the pattern of consumption of NO_2^- - N in fermentor is summarized in Table 3.12 extrapolated from the Fig 3.8. The shortest generation time noted was 15 days during the initial phase of growth. As the product build up accumulates, the generation time is seen to increase, probably due to product inhibition

Therefore, the generation time of the four consortia studied were fixed as follows: AMOPCU 1 : 10.5 hrs; NIOPCU 1 : 22.34 hrs; AMONPCU 1 : 21 hrs; NIONPCU1 a :15 hours. This information is authentic as the growth experiment was conducted under optimum conditions pertaining to each consortium. The product inhibition observed in all cases can be sited as a draw back in the batch mode of production of nitrifiers. This precisely indicates that a continuous mode

would be better to get good growth rate which will facilitate continuous removal of the products of nitrification. But in the present context such a system will not be useful because for the activation of bioreactor, dense suspension of nitrifiers is required as maximum quantity of the nitrifying consortium has to be immobilized on minimum substratum. To facilitate this, the diluted culture will have to be centrifuged to concentrate the biomass. This process will substantially add to the cost of production of nitrifiers and therefore the idea of centrifuging the culture was abandoned. Instead, it was decided to maintain the batch process as the mode of culture for the generation of the nitrifying biomass.

In literature, the estimated generation time for *Nitrosomonas* species is in the range of 8-36 hours and for *Nitrobacter* is in the range of 12-59 hours (Sharma and Ahlert, 1977). Interestingly the generation time of all the four consortia of nitrifiers fall within this range. Meanwhile, Skinner and Walker (1961) reported an extremely low generation time of 8 hours for *Nitrosomonas europeae*. At low substrate concentration the growth of nitrifying bacteria in batch systems follow the first order kinetics, i.e., the growth rate is proportional to the substrate concentration. Later, as the culture grows, it follows the zero order kinetics, i.e., the initial rate is independent of substrate concentration at high substrate concentration (Downing *et al*; 1964; Poduska and Andrews; 1975). This is because of the product inhibition where the ammonia oxidizers are inhibited by NO_2^--N and nitrate oxidizers by NO_3-N. Stein and Arp (1998) have reported that *N. europaea* lost an increasing amount of ammonia oxidation activity upon exposure to increasing concentration of nitrite, the primary product of ammonia oxidation. Since the substrate in all the cases were added exponentially, it never used to be a limiting factor.

3.3.6 Specific growth rate

The specific growth rate of ammonia and nitrite oxidizing consortia were determined and summarized in Table 3.13. Specific growth rate is inversely proportional to generation time and ranged from 0.033 to 0.063 L^{-1}.

Compared to heterotrophic bacteria, the specific growth rate is very low and is linked with the very long generation time as noticed in the fermentor under optimum growth conditions. The precise reason for a low specific growth rate and cell yield is the small energy gain obtained from oxidation of ammonia and nitrite (Prosser, 1989). On studying the growth parameters of *Nitrosomonas europaea* in the presence and absence of ammonia treated Vermiculate (AUT), Armstrong and Prosser (1988) noticed the specific growth rate ranging from 0.038 to 0.060 L^{-1} which is well comparable with the specific growth rate obtained with consortium in the present study. Ramachandran (1998), on working with consortia of ammonia and nitrite oxidizers developed from treated sewage recorded a specific growth rate of 0.029 L^{-1} and 0.041 L^{-1} respectively under optimum growth conditions in fermentor.

The above description clearly states that in all practical sense the consortia behaved almost similar to pure cultures. This includes the percentage of substrate oxidized out of the total quantity consumed, total biomass yield, alkalinity destruction by biomass yield, generation time, and specific growth rate. Clark and Schmidt (1966) stated that isolation of nitrifying bacteria in pure cultures is justifiably recognized as a difficult process and contaminants build up rapidly and reach high population densities under conditions specifically meant for chemolithotrophs. Moreover, as Gundersen (1966) pointed out that during mixed culture situation, nitrification reactions are frequently observed to proceed more rapidly in the presence of heterotrophic microorganisms than in pure cultures.

The consortia amplified were harvested and maintained at 4^0C without centrifugation and concentration of cells. This was done with an objective to make the technology economically viable and also to avoid any major change in composition of the culture under which they were growing. This would help the immobilization process in the reactor.

3.3.7 Phase contrast and Scanning electron microscopy observations

Phase contrast and scanning electron microscopy observations of the four consortia are presented in Fig 3.9 – 3.16. In all the cases uniformly the cells are aggregated to form zoogloeal mass protected with in a mucillagenous sheath, the glycocalyx. The cells are of different shapes and appearance indicating the mixed culture multispecies status of the consortium. Similar observations could be made by Ramachandran (1998) on studying the two nitrifying consortia isolated from sewage. An advantage of maintaining the consortia with glycocalyx is that the immobilization becomes much easier as the glycocalyx helps to attach the cells irreversibly to the plastic bead surface.

3.4 Summary

On mass producing the consortia in batch fermentor under optimum growth conditions, all the four cultures were found to be uniformly efficient > 87% of substrate consumed to the nitrification products. One of the problems encountered with the mass production of nitrifying consortia is the wall growth experience. This problem has to be addressed before commercializing the technology. To compensate the alkali destruction in AMOPCU 1 and AMONPCU 1, 94 and 52.25 ml each of 10% sodium carbonate was required per litre of the culture. Yield coefficient of all the four consortia was comparatively higher. The generation time of the consortia ranged from 10.5 to 22.34 hours. Specific growth of the consortia ranged from 0.033 to 0.063 L^{-1}, which is very low, compared with the heterotrophic growth. The consortia were found to be

with cells embedded in a mucilaginous sheath, capable enough to form biofilm on a new substratum.

Table 3.1 Substrate consumption and product build up of AMOPCU1 in fermentor

No:of days	Substrate consumed $NH_4(g.L^{-1})$	Product formed $NO_2(g.L^{-1})$
1	0	0
2	1.863	1.8975
3	3.726	3.7949
4	10.6605	7.5898
5	20.2215	16.0106
6	24.2658	29.2154
7	48.2104	41.0514
8	82.5214	73.9314
9	124.6144	107.1714
10	184.1674	165.213
11	234.4909	212.4314
12	282.0365	251.2114
13	329.582	289.9914
14	377.1286	328.7714
15	424.6735	367.5514
16	431.2135	406.3314
17	438.8012	432.629
18	461.9865	458.944
19	485.1718	485.259
20	510.9562	511.5914
21	543.7063	525.837
22	572.0757	540.083
23	603.726	554.322
24	637.0323	568.56
25	679.9952	582.802
26	691.892	597.04
27	703.7899	611.3114

Table 3.2 Substrate consumption and product build up of NIOPCU 1 in fermentor

No: of days	Substrate NO_2^--N consumed ($g\,L^{-1}$)	Product formed NO_3-N ($g\,L^{-1}$)
1	0	0
2	0	0
3	5.8447	4.5612
4	13.0764	10.3309
5	29.2406	17.8798
6	61.0734	43.3716
7	113.66	62.0325
8	530.3375	163.1915
9	542.634	264.3565
10	554.93	365.518
11	567.23	466.683
12	579.53	567.8445
13	591.83	669.0015
14	604.15797	676.4008
15	705.8585	698.97070
16	859.9678	860.710
17	961.1293	1122.8693
18	1062.2908	1284.6093
19	1163.4523	1446.3493
20	1264.6173	1483.245
21	1322.2957	1507.673
22	1409.8465	1670.8548
23	1497.3965	1834.0358
24	1584.9465	1997.2168
25	1672.4974	2160.3978
26	1760.04	2323.5788
27	1935.1513	2649.9457

Table 3.3 Substrate consumption and product build up of AMONPCU 1

No:of days	Substrate consumed (g L^{-1})	Product build up (g L^{-1})
1	0	0
2	0	0
3	0	0
4	0	0
5	4.242	0.5999
6	8.19	0.6333
7	9.398	0.6533
8	10.606	0.67330
9	11.814	0.7133
10	13.022	0.6933
11	14.28	0.7333
12	14.406	1.26655
13	14.532	1.7998
14	17.468	4.2996
15	19.2356	9.5687
16	22.8944	14.6985
17	30.6967	30.0303
18	40.6967	48.4285
19	49.5881	62.1271
20	65.8148	72.6927
21	80.7395,	89.991
22	103.2425	139.98
23	125.74558	189.98
24	148.248	239.98
25	170.7518	289.97
26	188.2188	326.634
27	205.685	344.41
28	216.735	362.186
29	238.5356	379.962

Table 3.4 Consumption pattern of NIOPCU 1 acclimatized to 15 ppt and name NIONPCU 1a

No:of days	NO_2 consumed ($g.L^{-1}$)	Product formed ($g.L^{-1}$)
1	0.7	6.4993
2	1.684	9.12354
3	2.1564	12.5404
4	5.6329	15.0004
5	10.499	21.6884
6	12.6655	26.124
7	22.1686	29.4578
8	40.5748	33.4587
9	42.6746	36.8799
10	28.2758	51.4025
11	72.6836	63.1544
12	89.7566	78.2504
13	92.6896	86.1327
14	112.6956	92.1546
15	132.5017	101.372
16	160.3775	112.254
17	179.6542	114.2546
18	181.4541	117.2154
20	192.7196	119.3342
21	222.6286	142.356
22	252.7376	176.7559
23	281.1801	303.4948
24	306.1561	313.0948
25	329.3753	422.8768
26	373.2208	466.2468
27	402.5846	503.707
28	447.6508	585.231
29	518.2117	612.321
30	609.5387	759.1
31	685.2401	812.456
32	822.1724	840.6414
33	963.3282	892.1
34	1025.1034	959.6418
35	1141.077	1281.911
36	1290.9497	1356.123
37	1360.4228	1575.52
38	1542.4346	1756.326
39	1649.4735	1783.486
40	1654.1966	1801.786
41	1679.9265	1867.8809
42	1755.8524	1905.0147
43	1844.0437	1943.5515
44	1927.6022	2013.1223
45	2053.0566	2158.57
46	2180.0774	2248.618
47	2313.9642	2327.5184

Table 3.5 Relationship between substrate consumption, product build up and biomass generated for different consortia in fermentor.

Consortia	Substrate (NH_4^+-N / NO_2^--N Consumption (gL^{-1})	Product (NO_2-N/ NO_3- N build up (gL^{-1})	Biomass generated ($g\ L^{-1}$)	%oxidation of NH_4^+-N/ NO_2-N	% used up for other purpose
AMOPCU 1	703.7899	611.3114	0.077	86.86	13.14
NIOPCU 1	5157.5934	4600.1298	0.068	89.19	10.81
AMONPCU 1	238.5356	379.962	0.3	159.29	*
NIONPCU 1 a	2313.9642	2327.5184	0.07	100.5858	*

* Product formed is higher than the substrate consumed suggesting the possibility of an extraneous source of NH_4^+-N and NO_2^-N respectively for AMONPCU 1 and NIONPCU 1 a.

Table 3.6 Enumeration of the four nitrifying consortia based on substrate consumption on the fermentor on attaining stationary phase.

Relationship between substrate oxidized and the number of cells generated	Quantity of substrate consumed μgL^{-1}	Quantity of substrate oxidized = product build up	No: of cells generated (per litre)
AMOPCU 1 3×10^4-1.2×10^5 cells/µg NH_4-N oxidized	7.04×10^8	6.11×10^8	1.833×10^{13}- 7.332×10^{13}
NIOPCU 1 $1\text{-}4 \times 10^4$ cells/µg NO_2-N oxidized	5.16×10^9	4.6×10^9	$4.6 \times 10^{13} - 18.4 \times 10^{13}$
AMONPCU 1 $3 \times 10^4 - 1.2 \times 10^5$ cells/ µg NH_4-N oxidized	2.39×10^8	3.8×10^8	$1.14 \times 10^{13} -$ 4.56×10^{13}
NIONPCU I a $1\text{-}4 \times 10^4$ cells/ µg NO_2-N oxidized	2.14×10^9	2.33×10^9	$2.33 \times 10^{13} -$ 9.32×10^{13}

Table 3.7 Alkalinity destroyed during mass production of AMOPCU 1 and AMONPCU 1 (6 – 7 mg alkalinity destroyed per mg NH_4^+-N oxidized to NO_2^- - N, EPA, 1975)

Consortia	pH Optima	Total NH_4^+-N removed gL^{-1}	Total alkalinity destroyed (g)	Volume of 10% sodium carbonate used to neutralize acid production (L^{-1})
AMOPCU 1	7.50	701.82	4210.92 – 5193.47	94.0
AMONPCU 1	8.5	151.82	910.92 – 1123.47	52.25

Table 3.8 Yield coefficient of the four consortia.

Consortia	Mass of cells generated mgL^{-1} (a)	Mass of substrate utilized mgL^{-1} (b)	Mass of product formed mgL^{-1} (c)	Y=a/b	Y=c/b
AMOPCU 1	77	703.7899	611.3114	0.1094	0.8686
NIOPCU 1	68.2	5157.5934	4600.1298	0.0132	0.8919
AMONPCU 1	300	238.5356	379.962	1.2577	1.5929
NIONPCU 1a	72	2318.9642	2327.5184	0.0310	1.0037

Table 3.9 Determination of generation time of AMOPCU 1

Quantity of NO_2^- - N on doubling its build up ($g.L^{-1}$)	Duration required for doubling NO_2^- - N output (hours)
0 - 1.89	12
1.89 - 3.78	10.5
3.78 - 7.56	51
7.56 - 15.12	19.5
15.12 - 30.24	18
30.24 - 60.48	39
60.48 - 120.96	70.8
120.96 - 241.92	62.4
241.92 - 483.84	175.2

Table 3.10 Determination of generation time of NIOPCU 1

Quantity of NO_2^- - N on doubling its consumption ($g.L^{-1}$)	Duration required for doubling the consumption (in hours)
0 - 1.95	23.92
1.95 - 3.90	22
3.90 - 7.8	32.32
7.8 - 15.6	21.92
15.6 - 31.12	21.92
31.12 - 62.4	22.96
62.4 - 124.48	33.08
124.48 - 249.60	43.2
249.60 - 499.20	86.4
499.20 - 998.4	124.8
998.4 - 1996.8	235.2
1996.8 - 3993.60	369.6

Table 3.11 Determination of generation time for AMONPCU 1

Quantity of NO_2^- - N on doubling its build up ($g.L^{-1}$)	Duration required for doubling NO_2^- - N out put (hours)
0 - 1.2	269.32
1.20 - 2.4	48
2.40 - 4.8	21.34
4.80 - 9.6	21.36
9.60 - 19.20	29.32
19.20 - 38.40	29.35
38.40 - 76.80	456.65
76.80 - 153.60	50
153.60 - 307.20	82

Table 3.12 Determination of generation time of NIONPCU 1a

Quantity of NO_2^- - N on doubling its consumption ($g.L^{-1}$)	Duration required for doubling the consumption in hours
0 – 0.7	24
0.7 – 1.4	15
1.4 – 2.8	37
2.8 – 5.6	19
5.6 – 11.2	30
11.2 – 22.4	43
22.4 – 44.8	50
44.8 – 89.6	70
89.6 – 179.2	156
179.2 – 358.4	173.14
358.4 – 716.8	130.86
716.8 – 1433.6	146.86
1433.6 – 2867.2	

Table 3.13 Specific growth rate of the four consortia
(Sp.growth rate hour $=\frac{0.693}{g}$, g = generation time)

Consortia	g (hours)	$H = \frac{0.693}{g}$
AMOPCU 1	10.5	0.063
NIOPCU 1	22.0	0.0315
AMONPCU 1	21.0	0.033
NIONPCU 1a	15.0	0.0462

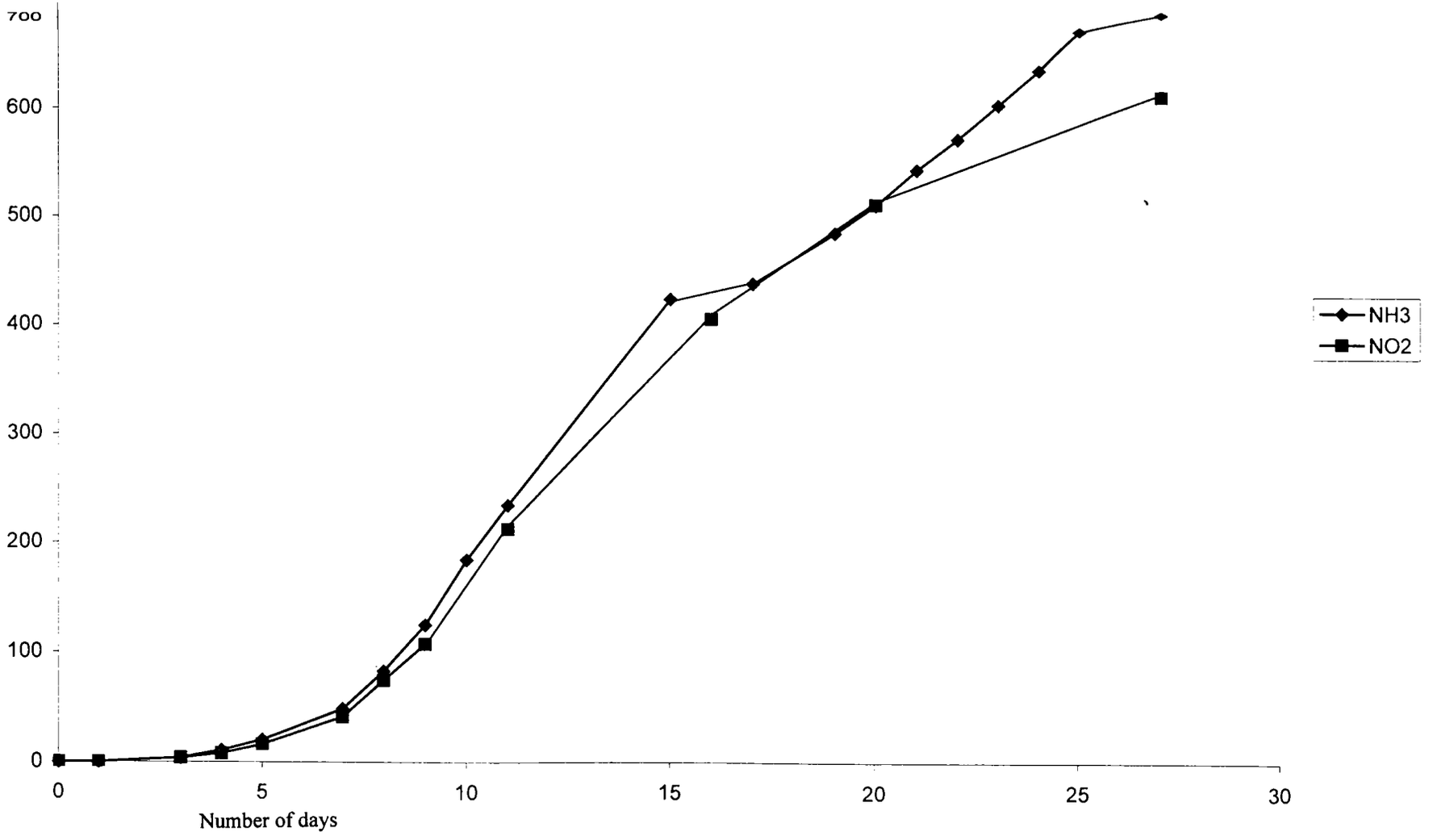
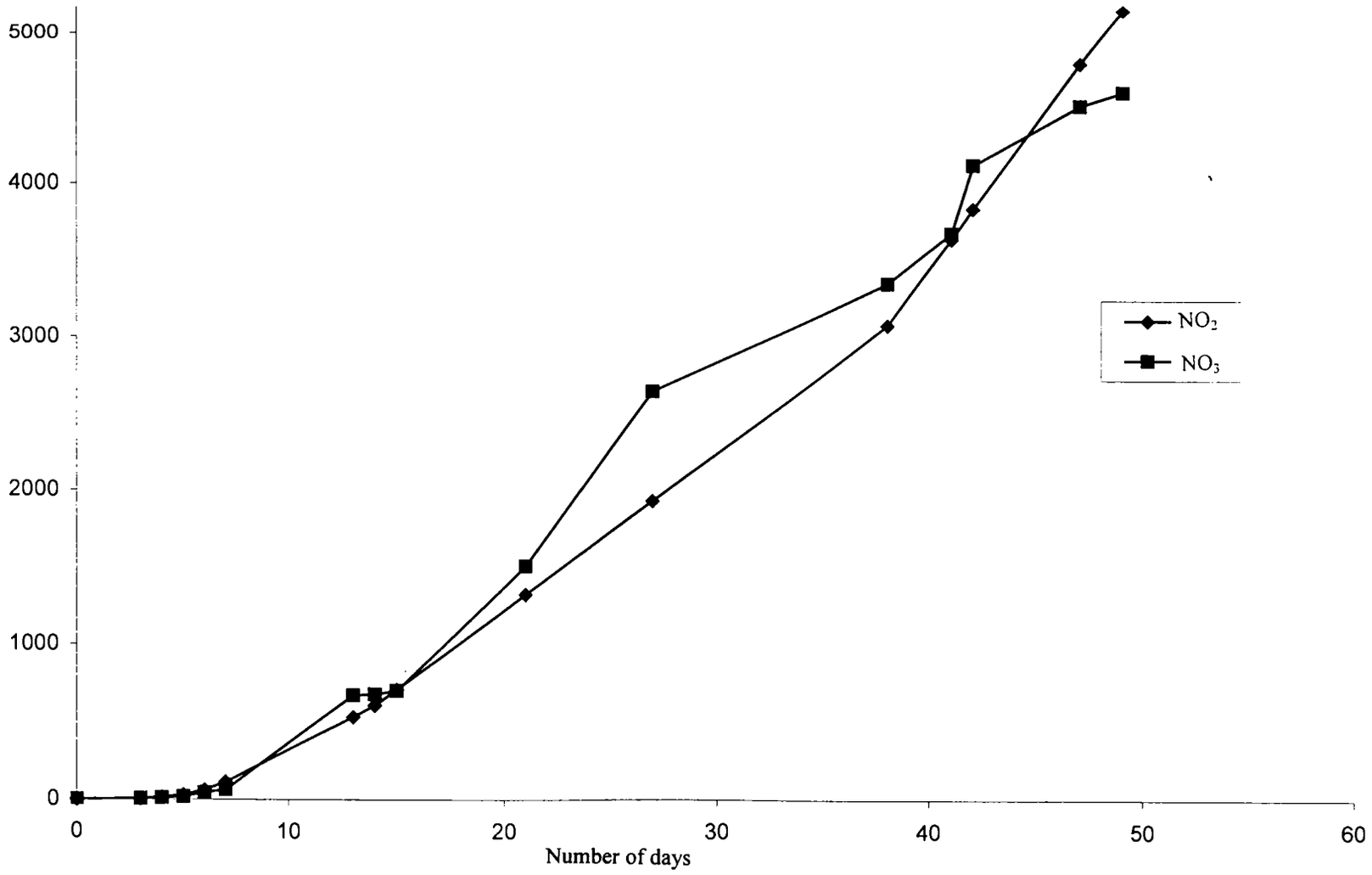


Fig 3.1 Growth curve of AMOPCU 1

NH_4: NH_4^+–N consumed (mgL^{-1})

NO_2 :NO_2^--N produced(mgL-1)



NO_2 : NO_2^--N Consumed (mgL^{-1}) Fig 3.2 Growth curve of NIOPCU 1

NO_3 : NO_3-N Produced (mgL^{-1})

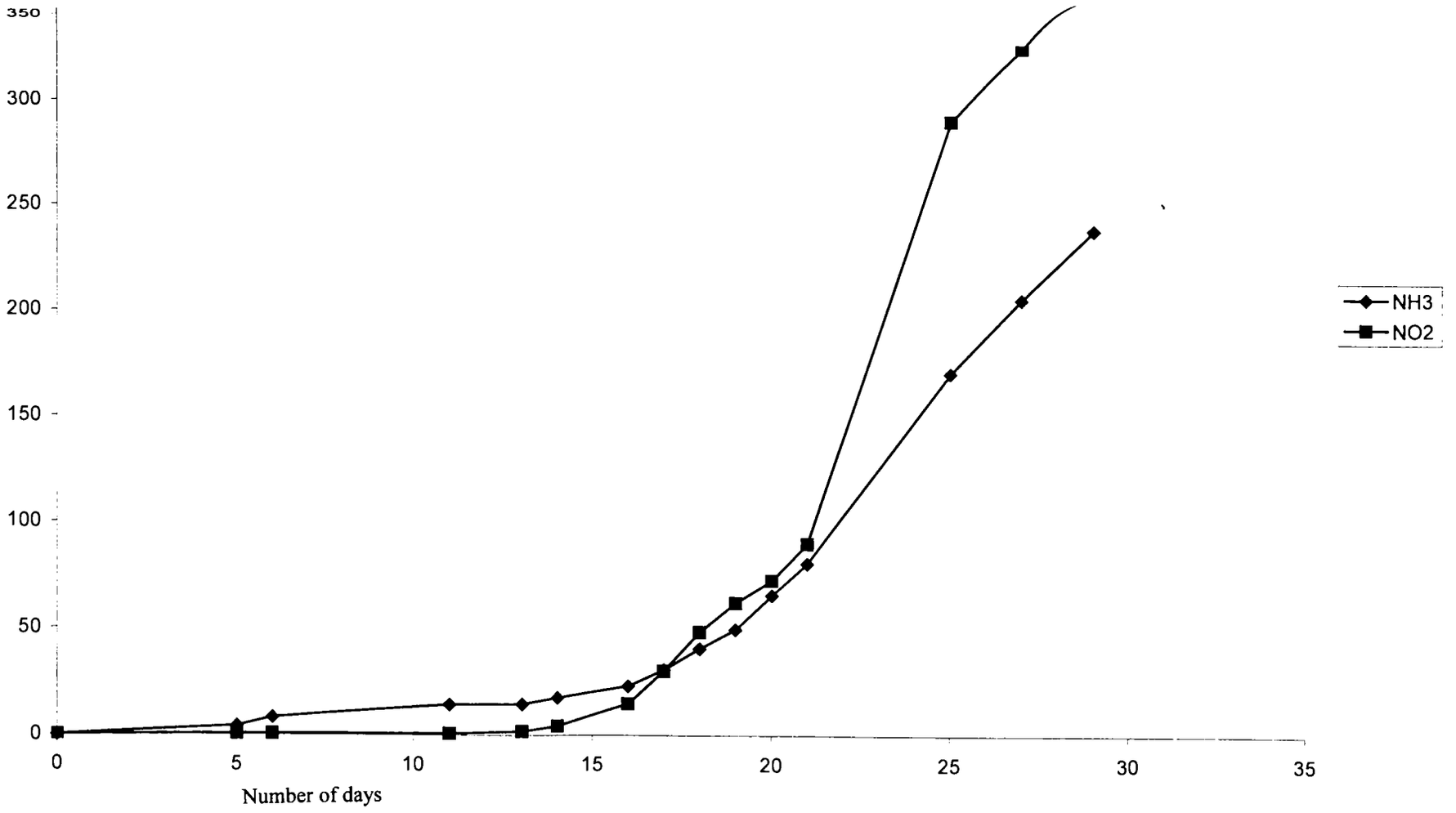


Fig 3.3 Growth curve of AMONPCU 1

NH_4: NH_4^+–N consumed (mgL^{-1})

NO_2 :NO_2^--N produced(mgL-1)

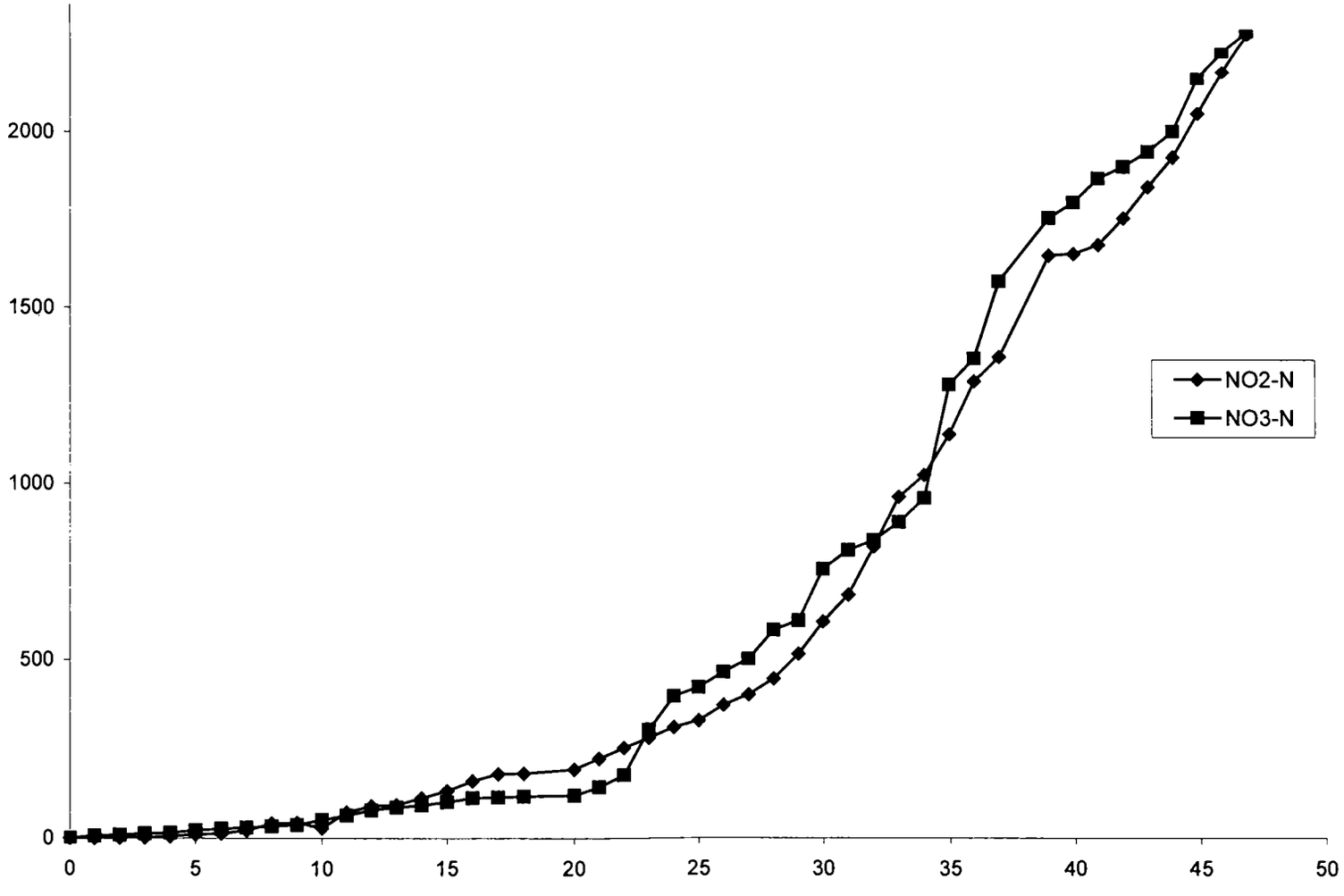
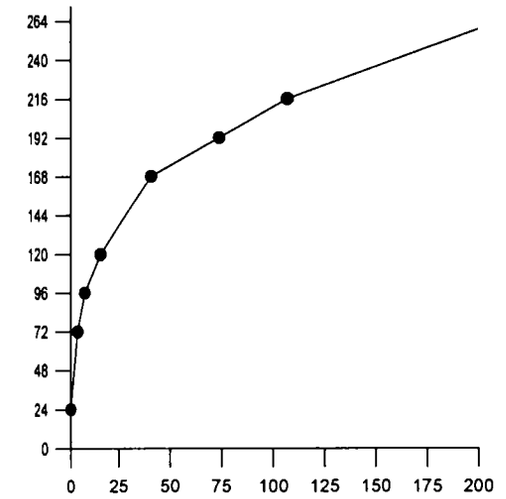


Fig 3.4 Growth curve of NIONPCU 1a

NO_2 – N: NO_2^- - N consumed (mg L^{-1}) NO_3 :NO_3-N produced(mgL-1)



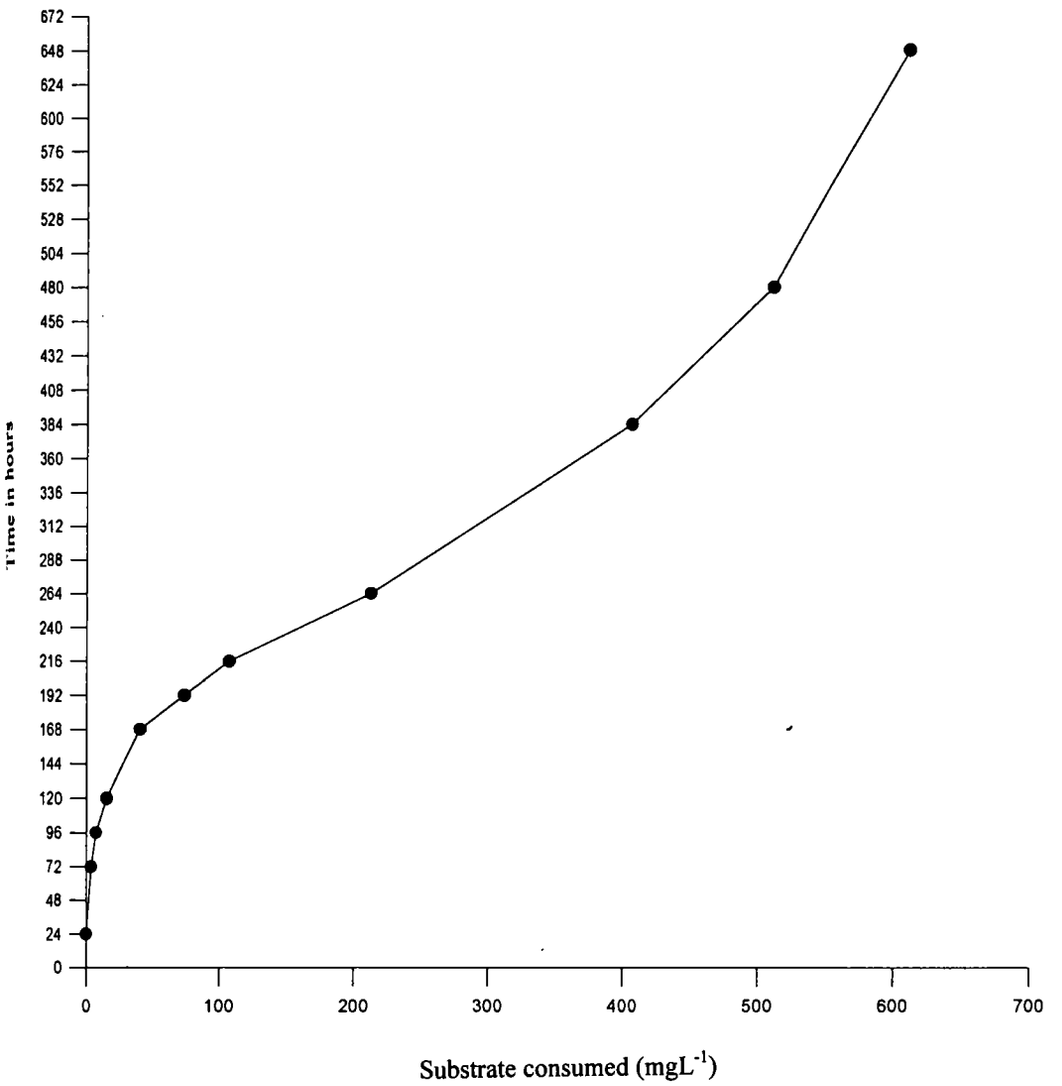


Fig 3.5 Determination of generation time of AMOPCU 1

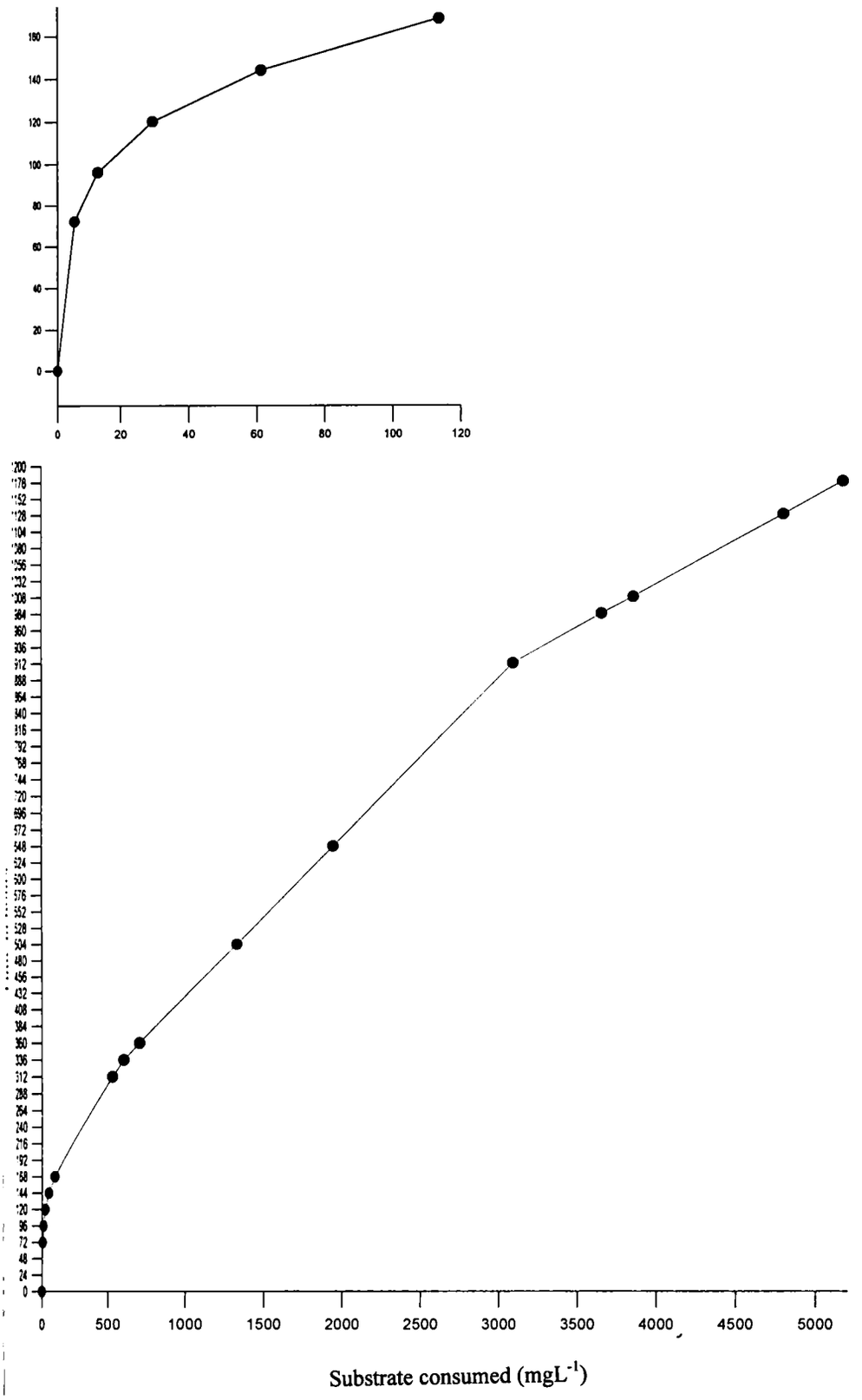


Fig 3.6 Determination of generation time of NIOPCU 1

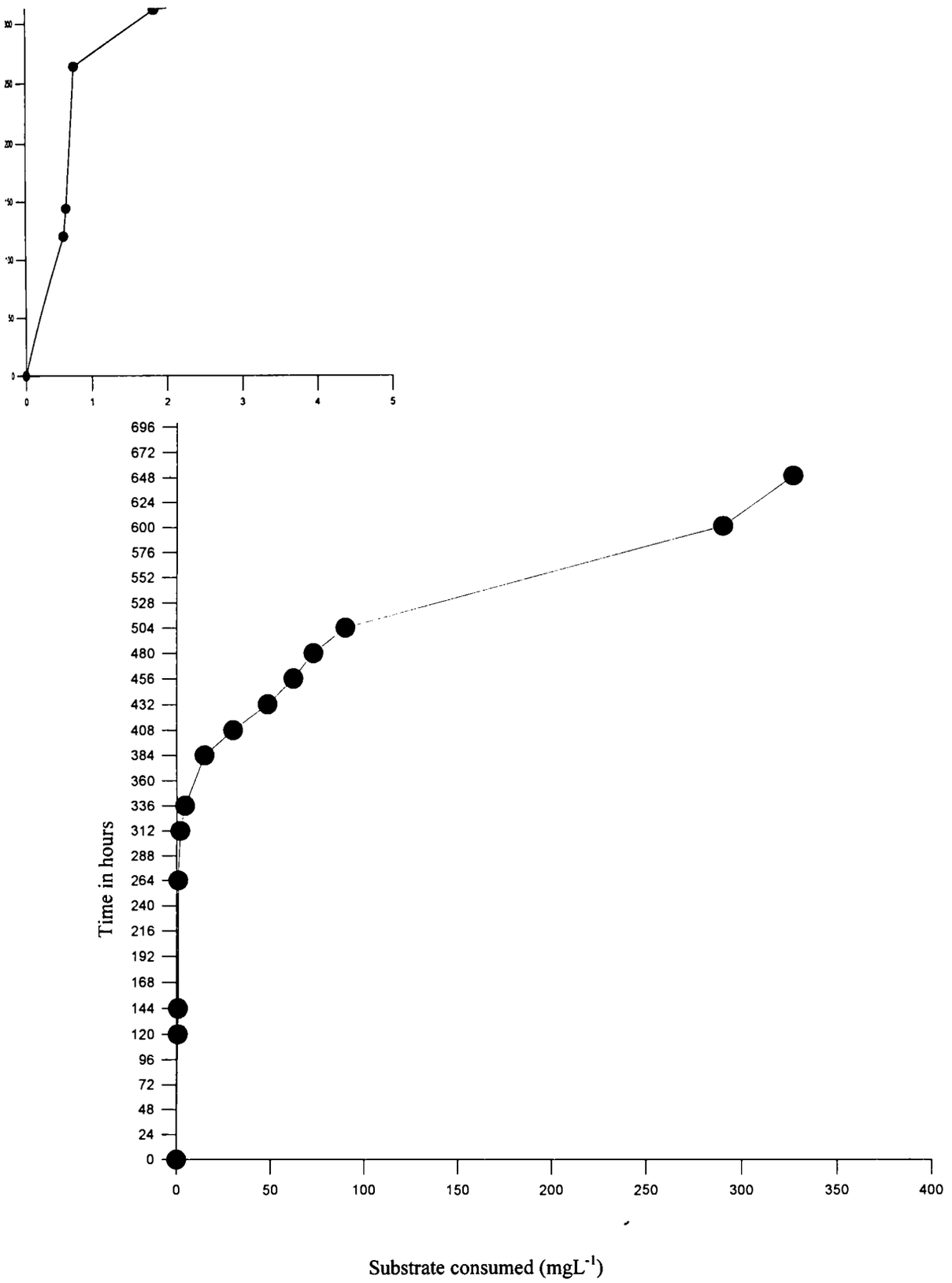
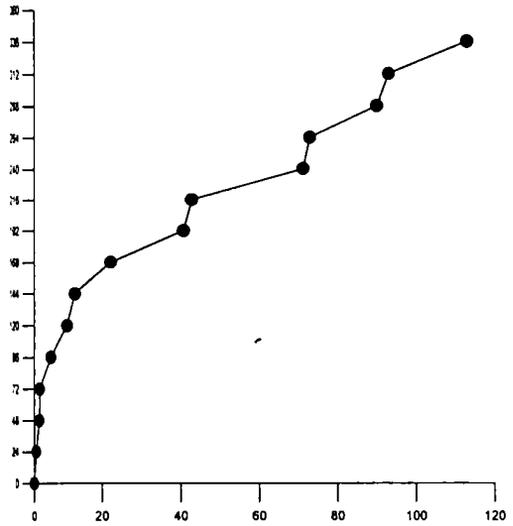


Fig 3.7 Determination of generation time of AMONPCU 1



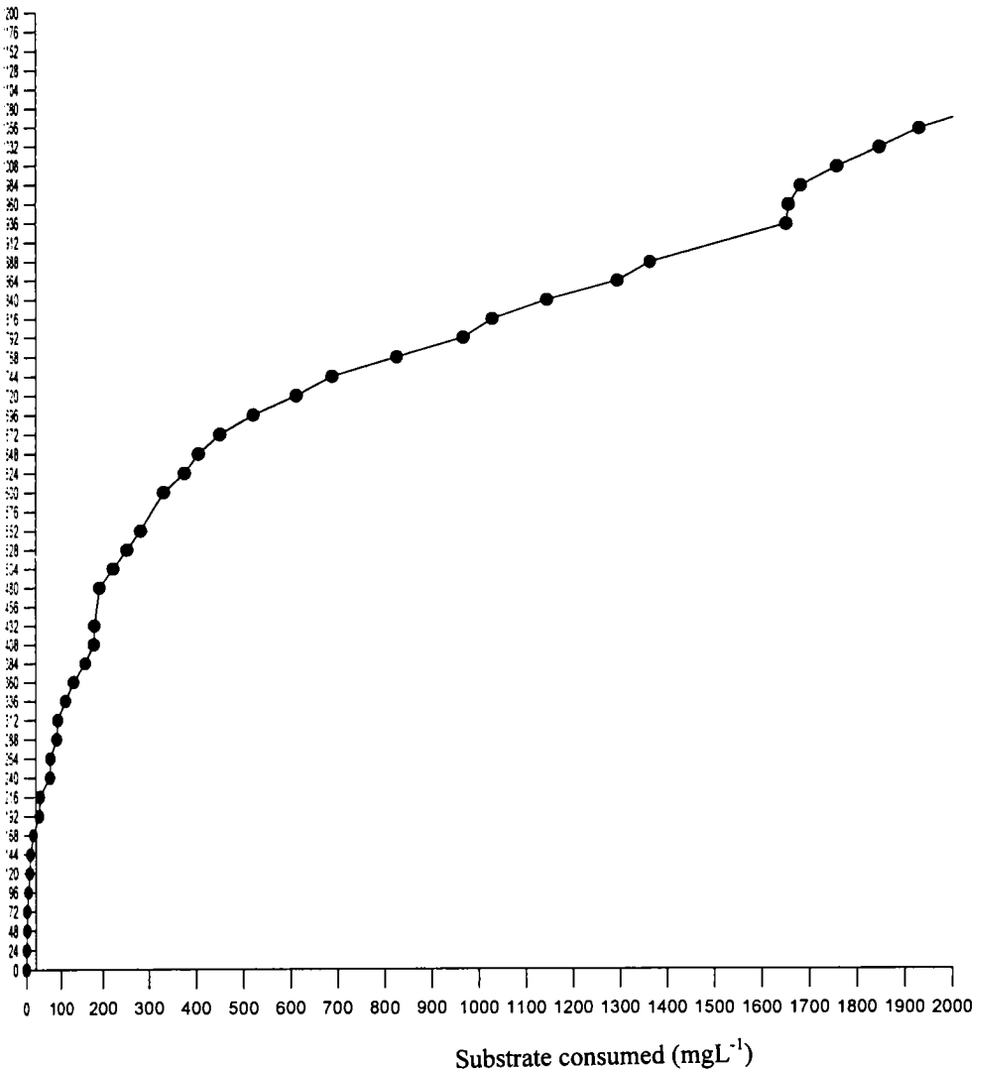
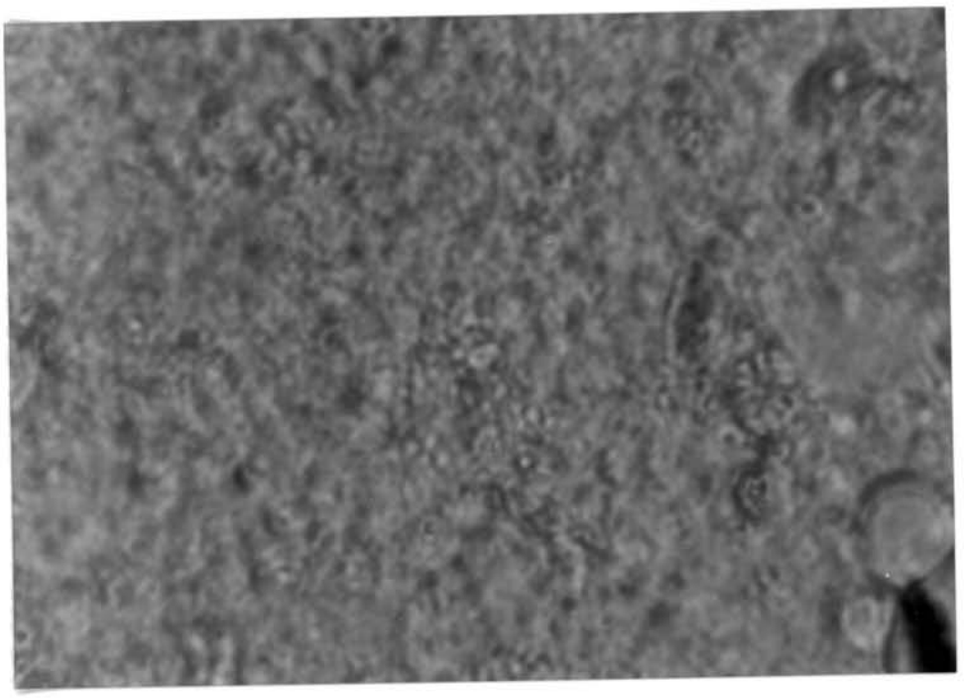


Fig 3.8 Determination of generation time of NIONPCU 1a

Fig 3.9 Phase photo micrograph of living cells of AMOPCU 1 suspended in seawa based medium (1000 X). Note the cells embedded in a mucilagenous matrix

Fig 3.10 Phase photo micrograph of living cells of NIOPCU 1 suspended in seawa based medium (1000 X) Note the cells embedded in a mucilagenous matrix



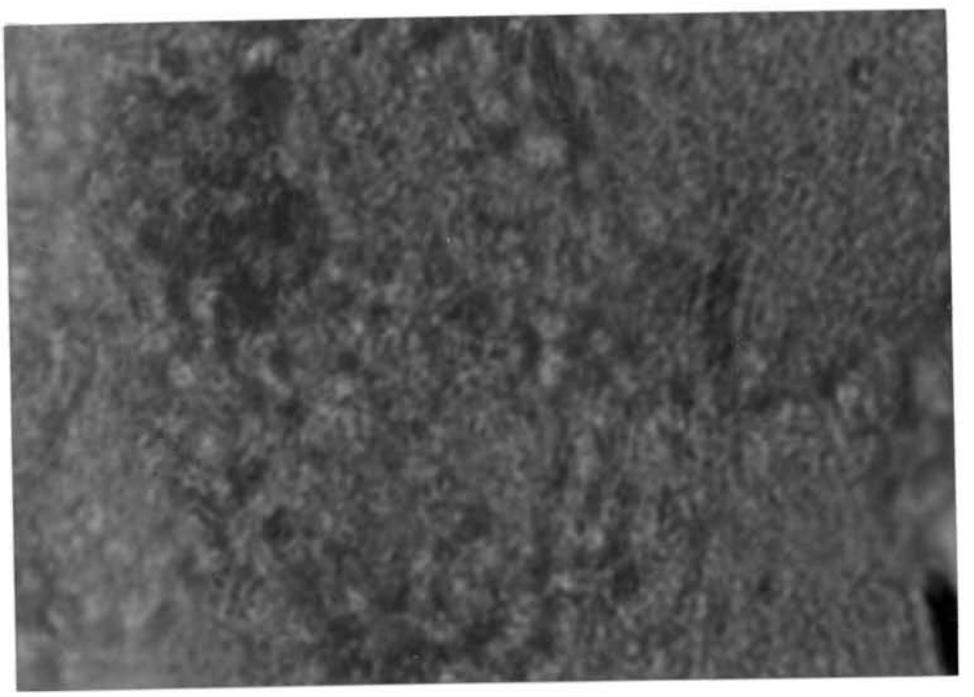
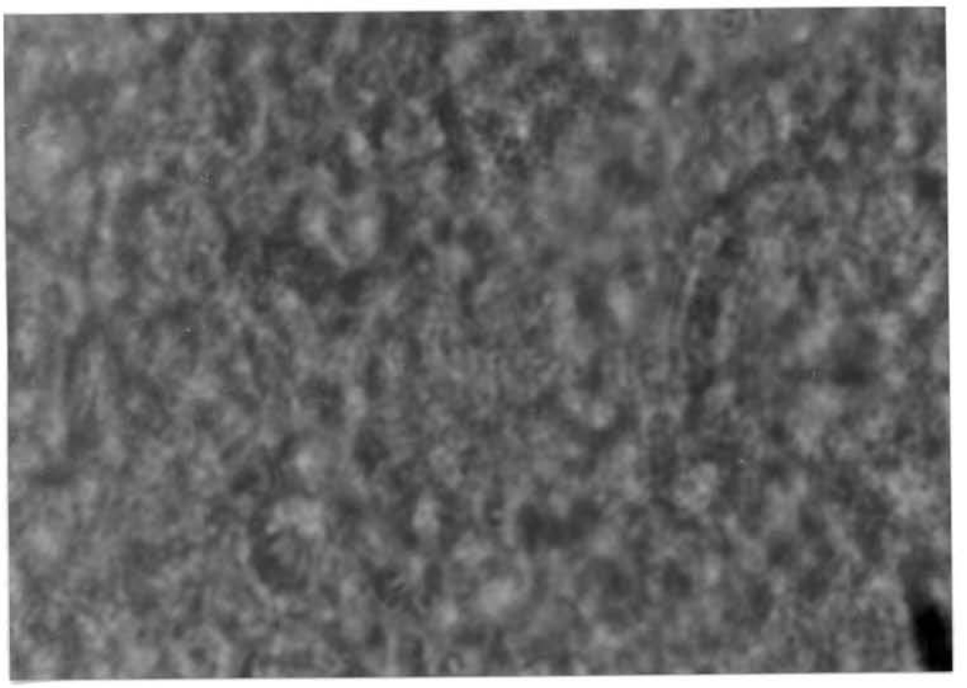


Fig 3.11 Phase photo micrograph of living cells of AMONPCU 1 suspended in seawater based medium (1000 X) .Note the cells embedded in a mucilagenous matrix

Fig 3.12 Phase photo micrograph of living cells of NIONPCU 1a suspended in seawater based medium (1000 X) .Thick walled cyst can be seen as seen in *Nitrosocystis oceanus* (Watson, 1965)



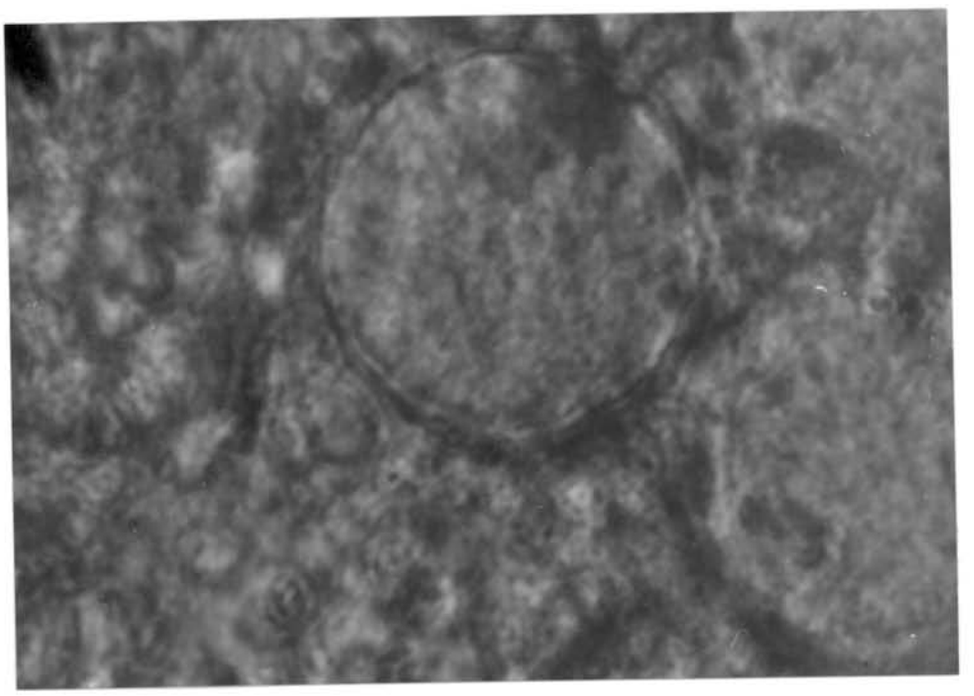
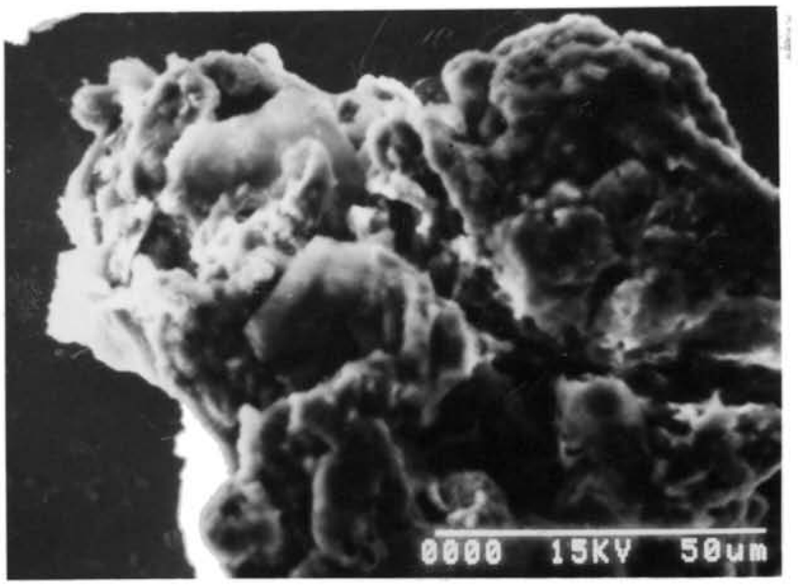


Fig 3.13 Scanning electron photo micrograph of AMOPCU 1
Cells are seen embedded in mucilagenous matrix

Fig 3.14 Scanning electron photo micrograph of NIOPCU 1
Cells are seen embedded in mucilagenous matrix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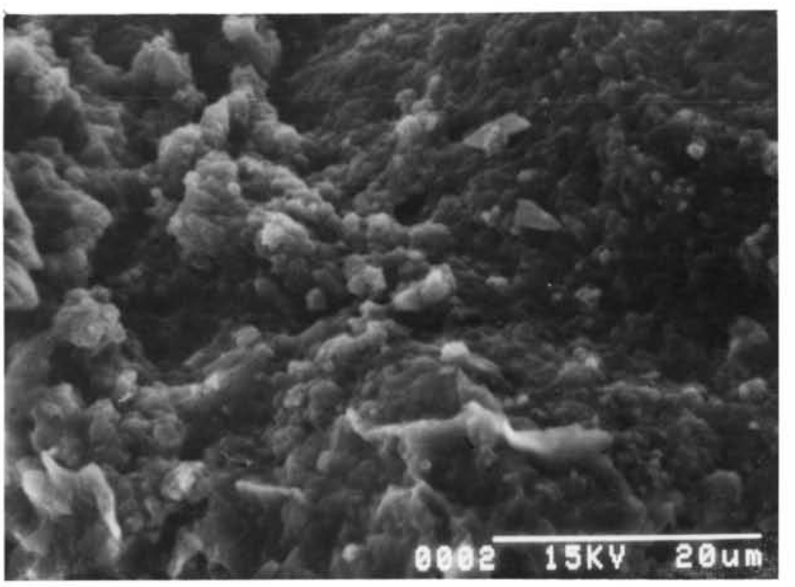
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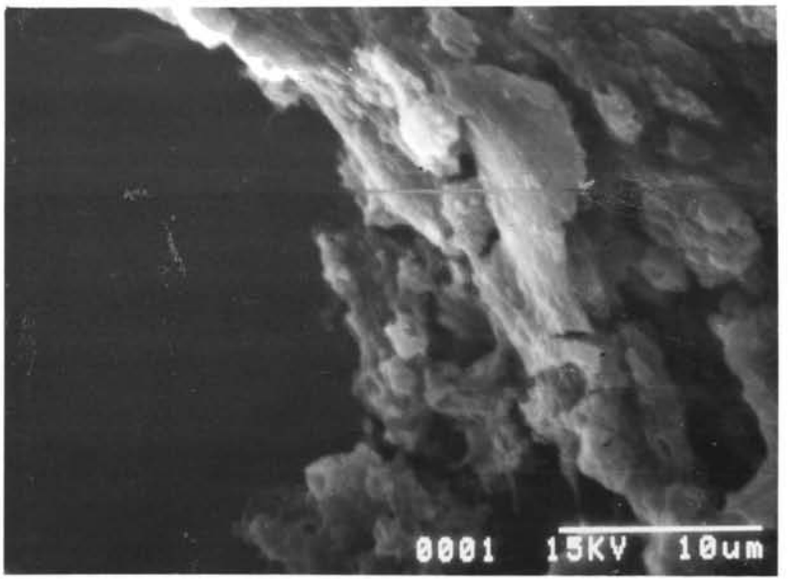
Fig 3.15 Scanning electron photo micrograph of AMONPCU 1
Cells are seen embedded in mucilagenous matrix

Fig 3.16 Scanning electron photo micrograph of NIONPCU 1a
Cells are seen embedded in mucilagenous matrix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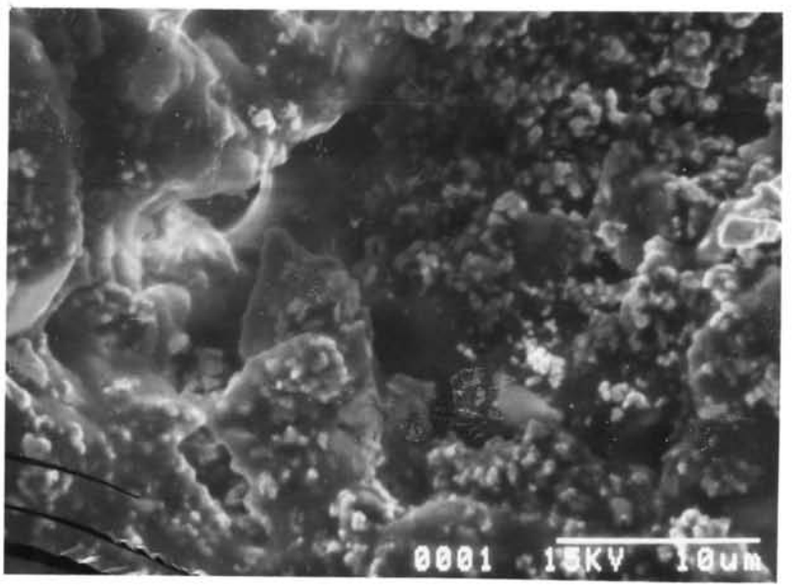
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CHAPTER 4

DEVELOPMENT OF BIOREACTORS

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DEVELOPMENT OF BIOREACTORS

4.1 Introduction

Ammonia is the main nitrogenous excretory product of prawn and is present in hatchery waters as a result of protein catabolism. Prawns digest the protein in their feed and excrete ammonia through their gills and in their faeces. Ammonia also enters the pond from bacterial decomposition of organic matter such as uneaten feed or dead algae and aquatic plants.

Total ammonia nitrogen (TAN) is composed of toxic un-ionized ammonia (NH_3) and non-toxic (ionized) ammonia . Only a fraction of the TAN exist as toxic (un–ionized ammonia) and a balance exists between it and the non-toxic ionized ammonia.

$$H + NH_3 \longleftrightarrow NH_4^+$$

The proportion of TAN in the toxic form increases as the temperature and pH of the water increases. For every pH increase of one 'unit', the amount of toxic un-ionized ammonia increases about 10 times. The concentration of un-ionized ammonia on either side of a tissue barrier at a given moment depends upon the pH of the water. Usually a gradient exists at these places where the pH of the extracellular fluid (water) and the intracellular fluid (blood) are not in equilibrium. When the pH of either fluid changes, there is a shift in the concentration of un-ionized ammonia on both sides of the barrier. The part of the tissue barrier with the lower pH attracts NH_3. Ionized ammonia (NH_4^+) is unable to pass tissue barriers. A decrease in dissolved oxygen increases the toxicity of un-ionized ammonia. An increase in the dissolved oxygen from 1.5 to 8.5 ppm at

a constant temperature of 19.8^{0}C reduced the toxicity of un-ionized ammonia. Thus maintaining high dissolved oxygen levels by aeration will slightly reduce the toxic effect of unionized ammonia.

When ammonia concentration becomes too high, fish are stressed, resulting in growth reduction. Ammonia is a major cause of death in unconditioned recirculating systems (Spotte, 1970). In general, the concentration of ammonia in solution (as total NH_4^+) should not exceed 0.1 ppm. Lethal levels for many animals are even lower and toxicity begins as low as 0.05 ppm. Un-ionized ammonia, even in minute concentration, damages the gill epithelium of fishes. When a sub-lethal long term exposure of salmon occurred, there was a reduction of growth rate and stamina. Exposure to ammonia caused gill hyperplasia, which was the precursor of bacterial gill disease. In healthy fish or prawn, culture pond ammonia levels should be zero.

Total ammonia nitrogen is converted to non-toxic nitrate via nitrite, which again is toxic to bacterial nitrification. Brown blood disease occurs in fish when water contains high nitrite concentrations. Brown blood cannot carry sufficient amounts of oxygen and affected animals can suffocate despite adequate oxygen concentrations in the water. The toxicity levels will probably change with water conditions like pH. The percentage un-ionized nitrite (HNO_2) increases at lower pH values. Un-ionized non polar forms of molecules traverse biological membranes more rapidly than their ionized counterparts and are more toxic to organisms. The 96 hour LC_{50} values of nitrite for crustacean larvae was 6 to 12 mg L^{-1}. Mortalities occurred in concentrations of nitrite as low as 3.3 mgL^{-1}, there is evidence of sublethal effects were incurred at 1.8 mg L^{-1} and growth of larvae in this concentration was retarded. Slow development and mortalities occurred at concentrations of 1.4 mg L^{-1}. Cessation of feeding and arrested development occurred when nitrite levels reached 1-1.5mg L^{-1}. The incipient lethal level i.e the nitrite concentration beyond which 50% of the population cannot live for a indefinite period was around 3mg L^{-1} for *Macrobrachium rosenbergii* larvae.

Thus the optimum parameters relating to nitrogen compounds in hatcheries are summarized below.

NH_4 (ammonium)	not greater than 15ppm
NH_3 (ammonia)	not greater than 0.1 ppm
NO_2 (nitrite)	not greater than 1 ppm
NO_3 (nitrate)	not greater than 20 pppm

Biological filtration is a series of steps in which nitrogenous organic compounds are mineralized, converted to inorganic nitrogen of successively higher oxidation states and finally removed from the solution by complete biochemical reduction and subsequent loss to the atmosphere or by incomplete reduction followed by assimilation in to plant tissues. Kholdebarin and Oertli (1977) proved that nitrification rates were enhanced in the presence of particles because of the physical attachment sites they provide. The increased surface area offered by the filter bed increases bacteriological growth and thereby activity, which occurs inside living films, attached to the walls, gravel and other solid surfaces. Kawai *et al* (1964) considered a filter bed to be conditioned when it had acquired sufficient nitrifying capability. This required approximately two months under warm water conditions. Kawai *et al.,* (1964) showed that the population of nitrifiers in a filter bed stabilized at approximately 60 days and Srna and Baggaley (1975) found that 40 days were required to generate a significant population of nitrifiers in a new filter. Based on test conducted, at 26^0C, Forster (1974) suggested that 30 days were necessary for a new filter bed to reach full nitrifying potential. Ammonia oxidizers reach maximum density after a month or so (Kawai *et al.,* 1964) under warm water conditions whereas nitrite oxidizers require approximately 2 months. They determined that the number of each group seemed to stabilize after reaching maximum density.

The time lag that biological filters require for getting established is the most glaring disadvantage for it. Spotte (1979) opined that the time lag in the filter beds can be reduced considerably by addition of bacteria already acclimated to aquarium conditions. Even pure cultures of nitrifiers show extended lag phases. So there is a need for a bioreactor which can be set up instantaneously as per demand and can hold nitrifying bacteria at very high density which can be used in the hatchery system for removing toxic ammonia and nitrite at a faster rate.

Steady state biomass concentrations can be obtained by combining bioreactors with filtration devices to retain biomass. Such retention cultures systems have been successfully applied in slow growing microorganisms including *Nitrosomonas* spp. Reactors in waste water treatment employ immobilized bacteria in order to retain slow growing bacteria in high flow systems or to provide dense biomass in a limited space. The immobilized cell systems offer highly accelerated reaction rates due to increase in cell density and since organisms are attached they operate at high operation rate without wash out. The inert solid supports in dilute media concentrate nutrients at the liquid solid interface and cells attached to the supports are exposed to higher nutrient concentration than existing in the bulk fluid. Thus, higher cell population and faster growth rates may be achieved.

Various methods of immobilization are in use like adsorption, covalent bonding, cross linking, entrapment and encapsulation. The criteria imposed for cell immobilization is the nature of application. Adsorption of cells is generally considered to be one of the easiest methods of immobilization. The early stages of bacterial adhesion can be described by Van der Waals forces of attraction and electrostatic forces of repulsion as formulated by DLVO theory. Van Loosedrecht *et al.*, (1997) showed that surface hydrophobicity plays a dominant role because the Van der Waals force of attraction increases with surface hydrophobicity.

Both ammonia and nitrite oxidizers form cell aggregates and appear enmeshed in exopolymers and they have a negative charge during their growth period. Even if supports are negatively charged they get attached to some other means other than electrostatic interactions.

For immobilizing nitrifiers, various polymeric supports like HDPS, HDPE, PP, PVC, PMMA were used by Souza *et al.*, 1997. PP exhibited higher rates of ammonia oxidation followed by other polymeric supports. In support selection, dissimilar behaviors are exhibited by the two groups of bacteria. This has been attributed to the fact that ammonia oxidizers have smaller hydrophobicity and smaller charge while nitrite oxidizers are more hydrophobic and less negative (Souza *et al*, 1997). To increase positive charge to surfaces and to provide ion- exchange capacity, to shorten the start-up phase several methods have been tried by Matsumura, 1997. He studied the effectiveness of a positively charged macroporous carrier 'AQUACEL' in the immobilization of nitrifying bacteria by adsorption. The carrier had broad pore size distribution and the large pores prevented the carrier surface from being fully covered with attached cells even after a long term cultivation. Stationary plastic packing material (nested sheets, random beads or saddles etc) were suggested by Alleman, 1991. Based on all these data, an immobilization media and methodology had to be worked out for nitrifiers.

A simple biofilter is some solid particulate media held in a container over which water-containing waste is pumped. Five different reactor configurations are described by Wheaton (1991) such as submerged filters, trickling filters, biodisks, biodrums and fluidized beds. Submerged filter consists of a container filled with porous media over which bacteria grow, which is non toxic and allows water to pass through easily. Common media types include rock, sand and plastic of various designs. The media is always submerged completely below the water surface. Based on the operational mode, submerged filter is divided into upflow, down flow and cross flow filters. Trickling filters are very similar to submerged

filters but the media are kept damp, but not submerged and water trickles down through the filter, so that the air circulates through the filter at the same time as water moves downward through the filter. Bio disks are composed of circular plates, each of which is attached to a common shaft. Plate spacing along the length of the shaft is minimized while maintaining enough space for wastewater to circulate between plates after a bacterial film has been established on both sides of each plate. The shaft passes through the center of each plate and is mounted in bearings attached to each end of the wastewater container. The mounting is located such that the plates are submerged in the wastewater to approximately one- half of their diameter. The shaft and plates attached to it are rotated by a power source. Flow through a biodisk is usually parallel to the shaft.

Biodrums look and operate similar to a biodisk except a cylindrical drum replaces the disks. The cylindrical drum surface is porous typically same type of mesh material and the drum is filled with solid media having a high specific area per unit volume. Plastic media, as rings or balls are usually used. Fluidized bed filter consist of a closed container that is partially with a particulate media, typically sand. Wastewater enters the filter through the bottom, passes through a distribution plate, moves through the media and exits from the top of the filter. During operation sufficient water flow is maintained to suspend the media particles in the upward flow. This is called fluidizing the bed.

The challenge before us is to produce a bioreactor that removes the ammonia and nitrite at the required rate, requires little maintenance, is cost effective and user friendly and can be integrated into the system in which it is operating.

Due to the time lag in getting a biological filter conditioned in hatcheries, the toxicity levels of ammonia and nitrite has always been a problem. Economic considerations always dictate increased animal loads in a system, which again aggravates the situation. The option left out is frequent water exchange. But the

threats associated with water exchange like invasion of disease causing organisms and pollutants into the hatcheries which may cause mass mortalities, force farmers to adopt a closed system where water once drawn in is let out after a full cycle. The issue addressed in this chapter is thus the development of bioreactors for nitrifying water in closed system prawn hatcheries with the nitrifying consortia developed.

4.2 Materials and Methods

This chapter deals with the designing fabrication, standardization and integration of various components of the nitrifying bioreactors meant for both penaeid and non-penaeid hatchery systems. The reactors have three major components such as a .Support material for nitrifiers, b. Cartridge and c. Shell of the reactor.

Depending on the requirement, two kinds of reactors have been envisaged. The first category is In Situ Stringed bed suspended bioreactor (SBSBR) and the second category, Ex Situ Packed bed bioreactor (PBBR). The first type of reactor is to be used in the larval rearing tank continuously during the larval rearing. The second category is meant for nitrifying fresh and spent water in the hatchery. These reactors have been developed separately for both penaeid and non-penaeid larval rearing systems where the salinity of the rearing water varies very much.

4.2.1 Support material for immobilization of nitrifiers

The bioreactors envisaged is based on immobilized cells on suitable support material (biofilms) through which water freely flows through, attaining nitrification. The extent of immobilization of bacteria on solid surface depends on hydrophobicity and electrostatic force of interaction between the bacterial surface and the solid surface. In biofilters traditionally several support materials such as sand grains, gravel, oyster shells, glass beads, plastic etc have been used.

Plastics in general have several advantages like inertness in aquatic system, lightness, hydrophobicity, easiness to mould into any shape, easy availability, easiness to handle and clean and above all are not expensive too. Therefore, different types of plastics coded as HA, LB, PC, PD, PE, PF, PG and AH were considered as support materials, to select the best one for each consortium is to be selected.

[Note: Application has been filed for granting patent to the bioreactor technology developed and the actual technical terms of the plastics used have been masked at the request of the funding agency]

4.2.1.a Designing and moulding of plastic beads

The above mentioned eight types of plastics were moulded into 5 mm diameter beads with a 2 mm hole at the centre with sparking on the surface. Spherical shape and sparking on the surface specifically served to increase the surface area and thereby the attachment sites. The hole at the centre facilitated arranging the beads in strings in SBSBR besides measuring the surface area.

4.2.1.b Preparative washing protocol

The beads were kept immersed in 0.1 N HCl for 3 hours and washed with 10% Extran, subsequently with tap water (5-10 times), and then with distilled water and was then air dried.

4.2.2 Development of an appropriate immobilization technique for each consortium

This is the crucial component of the bioreactor technology to be developed which depends on various intrinsic and extrinsic factors. The immobilization should be irreversible, non lethal to the consortium concerned, easy to carry out at

commercial level and should all the more be commercially viable. Therefore the experiments were programmed and executed with the above as prime objectives.

4.2.2.a Polyethylene imine treatment of plastic beads to enhance adsorption of nitrifiers

The extent of immobilization depends on hydrophobicity of surface and electrostatic force of interaction between the solid and bacterial surface (Souza *et al.*, 1997). Plastics are hydrophobic and bacterial surfaces are negatively charged. Therefore, if positive charge could be imparted to the bead surface, the process of immobilization could be made very fast and irreversible. To achieve this end, an ion exchanger, polyethylene imine (Matsumura *et al.*, 1997) was applied to impart the cationic characters to the substratum.

A 2% polyethylene imine (Sigma chemical Co., USA) was prepared in 50 numbers of the distilled water the plastic beads (Code PE) 50 numbers were immersed over night in this. Subsequently the beads were gently washed with distilled water. The treated beads were kept in the growth medium (50 mL) of Watson (1965) having salinity 30 ppt, pH 8.5, NO_2^--N 10 μg mL^{-1} inoculated with 0.5 mL consortium, NIOPCU 1 (0.0297mg dry weight). The preparation was incubated over rotary shaker at room temperature for 3 hours. The control set consisted of the same preparation with untreated beads. After the incubation period the beads were gently washed with sterile growth medium and transferred to fresh growth medium and from that point onwards consumption of NO_2^--N and production of NO_3-N were monitored both in test and control flasks.

4.2.2.b Toxicity of polyethylene imine on nitrifying consortium

This experiment was conducted to reveal the toxicity of polyethylene imine, if at all any, on nitrifying bacteria. For this different concentrations of polyethylene imine such as 0%, 0.01%. 0.05%, 0.075%, 0.1%. 0.15% and 0.2%

were prepared in 50 mL aliquots of the medium (Watson, 1965) and to each such preparation 0.5 mL consortium was inoculated in duplicates and incubated under obscurity in an orbital shaker at 100 rpm. Starting from zero, once in every 24 hour, concentration of NO_2^--N and NO_3-N were recorded.

4.2.2.c Toxicity of polyethylene imine treated PE beads on larvae

This experiment was conducted to evaluate the possible toxicity of polyethylene imine treated beads (PE) on the larvae of *M. rosenbergii*. The beads were treated as described under section 5.2.2. a and 100 such beads were introduced into a 2L basin with 500 mL sea water (15 ppt) having 5 day old larvae of *M. rosenbergii*. The control set consisted of the same experimental design with untreated beads. Both the sets were maintained on identical conditions with gentle aeration (0.5 L^{-1}), 28 ± 1^0C feeding the larvae with *Artemia* nauplii (10 *Artemia* per *Macrobrachium* larva.) This was maintained for 48 hours and mortality of larvae was recorded.

4.2.2.d Nitrifying potential of NIOPCU 1 suspended in the growth medium

Application of PEI to enhance immobilization did not yield promising results. In this context, it was decided to try with alternate methods of immobilization to sort out the one, which would be ideal. This experiment was conducted to assess the viability and nitrifying potency to of NIOPCU 1 after a prolonged period of storage at 4^0C. The consortium which was amplified in fermentor had been concentrated by centrifugation ,washed with growth medium and had been under refrigeration for more than 6 months.

A 0.5 mL aliquot of the consortium (NIOPCU 1) was suspended in 50 mL medium (Watson, 1965) and incubated under obscurity on a rotary shaker in

duplicate. Starting from zero hour, the nitrification was measured for 24 hours by monitoring this consumption of NO_2^--N and production of NO_3-N.

4.2.2.e Influence of plastic beads (PE) on the activity of free cells of nitrifiers

This experiment was conducted to assess the influence of the presence of beads on the nitrifying potential and nitrification of free cells while suspended in medium. In order to assess these 10 numbers of plastic beads (PE) were introduced into 50 mL medium supplemented with 0.5 ml NIOPCU 1 and incubated for 24 hours and NO_2^--N and NO_3-N availability were determined. The control set consisted of flasks without the beads, and the performance in both the sets was compared.

4.2.2.f Three hour immobilization of nitrifiers on beads

The concept of three hour immobilization of nitrifiers on the surface of plastic beads was imbibed from such similar works especially by Ramachandran (1998) who used the treated plastic beads for immobilizing nitrifiers by adsorption. In that study three hour exposure was sufficient to have satisfactory irreversible attachment. But in the present case PEI was primarily toxic and therefore just a contact period of 3 hours was doubted as the sufficient period for effective adsorption. To evaluate the efficiency of keeping the beads in contact with the cells for effecting attachment within a short period of 3 hours the present experiment was carried out. The nitrite oxidizing consortium, NIOPCU 1 and the growth medium according to Watson (1965) was used for the experiment.

An aliquot of 0.5 mL NIOPCU 1 (0.0297mg dry weight) was suspended in 10 mL medium and 10 washed plastic beads (PE) were introduced. The flask was placed under obscurity on a rotary shaker and for 3 hours. At the end of the period the beads were recovered washed gently with growth medium and resuspended in 10 mL aliquot of the growth medium and incubated under the

above described conditions for 24 hours. At the end of this period nitrification was recorded starting from the zero hour. A negative control with medium and plastic beads not exposed to nitrifiers was run in parallel.

4.2.2.g Experiment with an alternative immobilization medium

This experiment was conducted to examine the efficacy of an alternative solution, 2 % saline (i.e., 2 g NaCl in 100 mL distilled water), containing 10 μg mL NO_2^--N to achieve immobilization of nitrifiers on the plastic beads. Here 0.5 mL consortium (NIOPCU 1) was suspended in 10 mL saline along with 10 plastic beads (PE) and kept under obscurity for 3 hours on a rotary shaker. The beads were recovered and gently washed with saline and transferred to the growth medium containing 10 μg mL^{-1} NO_2-N. The controls included one set of beads exposed to nitrifiers in the growth medium and another set with beads not exposed to the nitrifiers. NO_2^- -N and NO_3 -N was monitored for 24 hours to record nitrification.

4.2.2.h Extent of attachment of nitrifiers during 3 hour immobilization

The three hour immobilization of nitrifies on plastic beads is followed by a step of gentle washing to remove loosely attached cells. If the nitrifiers were not attached irreversibly, this gentle washing would be well sufficient to remove the entire cells. To assess the extent of immobilization, which would take place with in a period of 3 hours, the present work, was carried out.

The exposure of beads to the nitrifiers was carried out as described above. At the end of the 3 hour period the medium which was used for washing the beads exposed to nitrifiers was recovered and kept for incubation, under the similar conditions for 48 hours and simultaneously, the beads recovered after washing were resuspended in the growth medium and kept for incubation under the same conditions for 48 hours for monitoring nitrification in both the sets. The negative

control was a set of flasks with beads alone without any exposure to the consortium. Meanwhile as positive control beads after exposure to consortium were recovered and introduced into fresh growth medium without washing and nitrification was monitored.

4.2.2.i Minimum time required for irreversible attachment of nitrifiers on beads

The foregoing experiments suggested that just three-hour exposure was not sufficient to facilitate irreversible attachment of the cells of consortium on the bead surface. Considering this, the present experiment was executed to determine the minimum time required for effecting irreversible attachment of cells on beads.

Four sets of flasks with 10 mL medium (Watson, 1965) were charged with 0.5 mL aliquots of the consortium 10 beads each were introduced . The flasks were incubated under obscurity on a rotary shaker. At the end of 3 hour incubation the beads were recovered from the first flask in the series gently washed with the growth medium and resuspended in fresh aliquot of 10 mL growth medium and kept for incubation under obscurity on a rotary shaker for 3 days. After 24, 48 and 72 hours, beads from flasks of the series were recovered and introduced in to fresh medium and the nitrification was assessed. In all cases the fluid, which was drained off after the stipulated period of immobilization was also incubated, and nitrification measured. The data generated was analyzed for the minimum time required for the irreversible attachment of cells onto the surface of beads.

4.2.2.j Response of the consortium NIOPCU 1 to eight different kinds of plastic beads

Beads (10 numbers each) prepared out of eight types of plastics coded as HA, LB, PC, PD, PE, PF, PG and AH were washed well following the procedure

described under section 4.2.1.b made ready for the experiment. The growth medium (Watson, 1965) was dispensed in 250 mL conical flasks (nine numbers) in duplicate and to each flask, 10 beads each were introduced. One of the flasks was maintained without the addition of beads that which served as control. From zero hour onwards once in 24 hours the nitrification was recorded once in 24 hours, the nitrification was recorded for 72 hours. The extent of nitrification was compared to analyze the response of the consortium to the type of plastic used.

4.2.2.k Finalization of an appropriate protocol for immobilization

Based on the experimental results obtained a protocol was developed for the immobilization of nitrifying consortia on the surface of the plastic beads.

1. The immobilization fluid would be Watson (1965) medium with the salinity 30 ppt for AMOPCU 1 and NIOPCU 1, and 15 ppt for AMONPCU 1 and NIONPCU 1 supplemented with NH_4^+ -N and NO_2^--N as the case may be.
2. An immobilization period of 3 days is fixed as the minimum.
3. No additives like PEI is required to enhance the extent of immobilization.
4. Beads after the three day period of immobilization are not rinsed .

Based on these observations the protocol was made very simple and less expensive.

The beads along with the consortium is incubated in the Medium1 (Watson, 1965) for 3 days under obscurity on a rotary shaker and afterwards are transferred to fresh medium and incubated for effecting nitrification.

4.2.2.l Selection of appropriate support material for each consortium

The medium according to Watson (1965) was used as the immobilization medium containing 10 μg mL^{-1} NH_4^+-N / NO_2-N and 2 μg mL^{-1} PO_4-P. The

medium prepared in 50 mL aliquots was taken in 250 mL conical flask in triplicate for each type of bead. The other growth conditions such as pH, temperature and salinity were the ones optimum for each type of consortium which was as follows: AMOPCU 1 : pH 7.5, temperature 28^0C, salinity 30 ppt ; NIOPCU 1 : pH 7.0, temperature 28^0C, salinity 25 ppt ; AMONPCU 1 : pH 8.5, temperature 28^0C, salinity 10 and NIONPCU 1 : pH 4.0, temperature 45^0C, salinity 5 ppt . Ten beads each for each type of plastic were introduced into the flask and inoculated with the consortium which were generated in fermentor but not concentrated by centrifugation. It has to be noted that in all the previous experiments consortia, which were centrifuged and washed, were used. This change was brought in, considering the cost involved in centrifugation where the technology is commercialized and also the logical and scientific soundness in maintaining the culture without being disturbed by centrifugation and washing. A negative control was run with each set of plastic with out inoculum. The flasks were incubated over rotary shaker at room temperature of 28 ± 0.5^0C in dark. Starting from the point of inoculation, once in every 24 hours consumption of NH_4^+-N and production of NO_2^--N in the case of ammonia oxidizing consortia and consumption of NO_2^--N and production of NO_3-N in the case of nitrite oxidizing consortia were determined for 3 days. After completion of the immobilization period, the medium was drained off and the beads were transferred to fresh aliquots of 50 mL medium having the same composition and incubated for prolonged periods under the same conditions with daily monitoring of nitrification till considerable activity could be noticed.

The type of plastic in the presence of which highest percentage consumption of NH_4^+-N and production of NO_2^--N and consumption of NO_2^--N and production of NO_3-N obtained was demarcated as the best one which favored better attachment of nitrifiers and subsequent nitrification; such beads were segregated for the development of appropriate cartridges for the

bioreactors. Along with this, cost of the type of plastic used and easiness to mould these also have been considered for the final selection.

4.2.3 Designing and fabrication of Stringed bed suspended bioreactor, SBSBR

The reactor has four components such as,

1. The outer fiberglass shell of 10 cm^3 with conical bottom and perforations of 5 mm diameter at the top on two sides just beneath the margin (Fig. 4.1).
2. The inner cartridge comprising of a Perspex frame work and plastic beads on strings. The cartridge has a filter plate both at the top and bottom (Fig. 4.2, 4.3, 4.4). The filter plates are specially designed to have slits of 5 mm width and 10 to 15 mm length spaced equally. In between the rows of such slits pinholes are drilled to permit threads bearing strings of beads. The central opening is the space for accommodating the central air lift pump. The above cartridge is inserted into the shell and a black perspex lid is placed on top (Fig 4.5). The completed reactor can be suspended from a float through a string, which can be attached to the corners of the reactor. When the air lift pump is operated, water enters the reactor through the perforations on top margin of the shell and passes through the central pipe. The perspex framework of cartridge is made in such a way that larvae, plankton and food particles when entered pass through the airlift pump without mutilation and damage. The reactor cartridge has a surface area of > 684 cm^2 with full complements of beads. The reactors with ammonia oxidizing consortia are with beads of plastic coded as PE and the ones with nitrite oxidizing consortia are with the beads of plastic code as LB.

4.2.4 SBSBR activation and assessment

The reactors were separately activated using all the four consortia. Based on the unit nitrifying activity of each consortium, the quantity of inoculum used for attaining 500 units of activity for each consortium to be given for each reactor with respect to each culture was already worked out and was as follows:

AMOPCU 1	: 13.4 ml	;	(UNAa: 37.34 ± 0.29)
NIOPCU 1	: 14 ml	;	(UNAn: 35.36 ± 7.9)
AMONPCU 1	: 7 ml	;	(UNAa: 71.39 ± 0)
NIONPCU 1a	: 36 ml	;	(UNAn: 13.99 ± 0.9)

Uniformly all reactors received 500 units of consortium.

For the activation, reactors were put in an activation mode which consisted of a serological water bath with the temperature set at the optimum required such as for AMOPCU 1 28^0C, NIOPCU 1 37^0C, AMONPCU 1 28^0C and NIONPCU 1a 37^0C. Other optimum conditions given were pH and salinity of the immobilization medium adjusted to 7.5 and 30 for AMOPCU 1, 7 and 25 for NIOPCU 1, 8.5 and 10 for AMONPCU 1 and 7 and 15 for NIONPCU 1a respectively. Uniformly in all instances 10 μg mL^{-1} NH_4^+-N / NO_2^- -N and 2 μg mL^{-1} PO4^{--}P were also provided. At a time, for one consortium, 6 reactors were kept in the activation mode (4 tests and 2 control) with the lid open and the water level in the water bath filled to the level of holes on the sides of the reactor. The above described quantity of the consortium was given to attain 500 units in each reactor, aerosol arrestors were fixed and filtered air from a compressor was passed through the air lift pump using a specially designed air sparger in glass, at a rate of 1 litre min^{-1}. The initial substrate / product levels were estimated colorimetrically and the nitrifying biomass gravimetrically. For the latter, immediately on inoculation 10 mL medium with the bacterial suspension was withdrawn and passed through preweighed syringe type membranes of 0.22 μ

porosity and after drying at 80^0C in oven overnight the weight of the biomass was determined from the difference in weight. During the activation period of 3 days, the residual substrate and product level were monitored. Once the cells were found to have attached to the beads as evidenced by the disappearance of biomass from suspension and considerable amount of substrate utilization and product build up, could be confirmed the medium from the reactor was drained off and filled with fresh medium. The operation continued by passing air through the air lift pump. The activity of attached nitrifiers in terms of substrate consumption and product build up were monitored for another 3 days.During this period evaporation loss was compensated by adding autoclaved distilled water.

On the third day the aerosol arrestors were removed and the reactors were closed with black lid and suspended in plastic tanks with 5 mL each sea water supplemented with 10 $\mu g\ mL^{-1}$ NH_4^+-N / NO_2^- - N as the case may be. Here field conditions were maintained with the salinity adjusted to either 30 ppt or 15 ppt as is the case for penaeid or non-penaeid hatcheries. No adjustment of pH or temperature was made and only daily monitoring of substrate and the product were carried out to assess the activity and functioning of the reactors.

4.2.5 Field trial of *in situ* SBSBR in penaeid hatchery

The reactors after activation in the activation mode were ready enough to be moved on to the field (Fig 4.6). Transportation of the reactors was carried out "water-in" in a polythene bag . On reaching the site they were suspended in water slowly and allowed to hang on the float. The air tubing was connected to the main air supply of the hatchery and the airflow rate was regulated at the rate of 1 litre per minute.

The experimental design consisted of three oval shaped fiberglass tanks of 1000 mL capacity . Two spawners each were introduced into each tank named as A, B and C. Two ammonia oxidizing reactors and two nitrite oxidizing ones were

deployed in the tank A and the tank B (Fig. 4.7) and C were treated as controls. Soon after spawning, the spawners were removed and the hatching was monitored. In tank A and C complete hatching of eggs was recorded and at the same time in the tank B hatching was totally absent and hence the tank was abandoned. The nauplii in tank A and C were 100940 and 84280 respectively and quantity of water was around 600 L. Henceforth every day NH_4^+-N, NO_2^--N and NO_3-N were determined along with an estimation of the total larval population. The systems were monitored for 17 days till the larvae reached PL 7 stage.

4.2.6 Designing and fabrication of *ex situ* packed bed bioreactors PBBR

The *ex situ* packed bed bioreactors are with a base of 30 cm^2 and an overall height of 45 cm. A perforated base plate, fixed with 30 cm long and 2 cm diameter, 9 PVC pipes at 10 cm equidistant, is positioned at the base of the reactor on 5 cm long PVC pipes (Fig. 4.8 and 4.9). An inlet pipe of 2 cm diameter is fixed 35 cm up from the base of reactor shell which is made of fibre glass. An outlet pipe of the same diameter emerges from the base of the reactor, which bends upward and discharges at 35 cm from the base. Each pipe fixed on the perforated plate (9 numbers) function as an airlift pump when air passes through. Each 10 cm^3 area surrounding the airlift pump can be designated as an aeration cell when packed with the plastic beads selected for each consortium (PE for ammonia oxidizing consortium, LB for nitrite oxidizing). The reactor has a surface area of >47100 cm^2 on filling with beads.

The *ex situ* PBBR has to be integrated to the water storage facility of the hatchery system. Generally such storage facilities may not be available in all hatcheries and even if available they may not be having the required configuration to be used for getting integrated with the bioreactor developed. Therefore it was decided to offer the *ex situ* PBBR as a package along with the design for water storage facility and with a recirculation system. In this package (Fig 4.10, 4.11), there is an overhead tank(A) which opens to a tank kept at ground level (C).

From the bottom of the tank C an outlet pipe (D) connects to the reactor F_1, which is with ammonia oxidizers. An outlet pipe from this reactor connects to the second reactor F_2 that is with nitrite oxidizers. This reactor empties into the collecting tank, K from where it can be pumped back to the overhead tank or can be used for larval rearing.

4.2.7 Activation of *ex situ* PBBR

To fill up each reactor about 60,000 beads were required. The beads were subjected for a washing protocol as described under section 4.2.1.b and the reactor F_1 was filled up with the category of beads coded as PE and the reactor F_2 was filled with LB. In order to activate the reactor assembly for penaeid hatchery system the reactor F_1 was charged with 266.01 mL AMOPCU I and the reactor F_2 was charged with 268.00 mL NIOPCU I which uniformly supplied 10,000 units of inoculum. The reactor F_1 was filled up with 35 litres of autoclaved seawater having 30 ppt with pH adjusted to 7.5, supplemented with 10 $\mu g\ mL^{-1}$ NH_4^+-N and 2 mg L^{-1} PO_4-P. The reactor F_2 was filled up with 30 litres of autoclaved seawater at 25 ppt with pH adjusted to 7 supplemented with 10 $\mu g\ mL^{-1}$ NO_2^--N and 2 mg L^{-1} PO_4-P. No temperature regulation could be given in both the reactors. Through airlift pumps one litre air per minute was passed through to effect adequate circulation and aeration to the reactor bed. The reactors were covered with a black perspex lid to prevent sunlight.

The reactors for non-penaeid hatchery system were activated as follows. The reactor F_1 was charged with 140 mL AMOPCU 1 and reactor F_2 with 714 ml NIONPCU 1 a to supply 10,000 units of consortia uniformly. The reactor F_1 was filled up with 35 litres of autoclaved sea water at 10 ppt with pH adjusted to 7 supplemented with 10 $\mu g\ mL^{-1}$ NH_4^+-N and 2 mg L^{-1} PO_4-P. The reactor F_2 was filled up with 35 litres of autoclaved sea water at 15 ppt with pH adjusted to 7 supplemented with 10 $\mu g\ mL^{-1}$ NO_2^--N and 2 mg L^{-1} PO_4-P. Here also no temperature regulation could be given in both the reactors. Through the airlift

pumps one litre air per minute was passed through to effect adequate circulation and aeration to the reactor bed. The reactors were covered with a black perspex lid to prevent sunlight.

Assessment of the extent of attachment of nitrifiers onto the beads was made by measuring the disappearance of cells from the immobilization fluid in suspension gravimetrically as described under section 4.2.4 in *ex situ* PBBR. Starting from the zero hour once in 24 hours disappearance of NH_4^+ -N and building up of NO_2^{--} -N in the case of reactor F_1 and the disappearance of NO_2^{--} -N in the case of reactor F_2 were monitored regularly and the activation of the reactor was judged based on the above parameters. After 3 days, the entire fluid was drained off and filled with fresh seawater having 30 ppt salinity, with no adjustment in pH and substrate concentration of 10 μg mL^{-1} and the monitoring of their performance was carried out for another 3 days. On confirming that the reactors have become active, the valve B of the overhead tank can be opened and the water to be nitrified can be allowed to flow down and pass through the reactor.

[**Note:** The designs of the above category of reactors SBSBR and PBBR and the process developed have been approved by Department of Biotechnology, Govt. of India to the patenting office for granting Indian patent]

4.3 Results and Discussion

4.3.1 Effect of polyethylene imine treatment of plastic beads to enhance adsorption of nitrifiers

Effect of PEI treatment of beads on nitrification to enhance adsorption of nitrifiers is summarized in Table 4.1. During the 3 hour immobilization phase, eventhough NO_2^{--} -N consumption was found to be higher the PEI treated beads compared to untreated beads, NO_3-N build up was not observed with PEI treated ones. Meanwhile substantial quantity of NO_2^{--} -N was build up during the short

period of 24 hours in the set of flasks with the beads not treated with PEI. In the second phase of experiment when the beads were recovered and transferred to fresh medium, the beads without PEI treatment was found to exhibit NO_2^--N uptake than the one with PEI treatment. But in none of the cases, NO_3-N build up could be seen.

It could be inferred that PEI treatment was not in any way helping the immobilisation of nitrifiers by adsorption on the surface of the beads. Moreover, there is indication of a certain level of toxicity too on the nitrifiers. The experiment has suggested that mere exposure of beads to nitrifiers in suspension for 3 hours will not be sufficient for the effective attachment of cells on the bead surface.

4.3.2 Toxicity of polyethylene imine on nitrification

Effect of PEI treatment of beads on nitrifying bacteria in terms of its nitrifying potential is summarized in Table 4.2. On observing the activity of the consortium over a period of 24 hours in the presence of varying concentrations of PEI, the consortium was found to be active only in the medium without PEI and the lowest concentration of 0.01% in terms of NO_2^--N consumption and NO_3-N build up. Here also the activity was less in the latter case compared to the former. Meanwhile there was substantial disappearance of NO_2^--N in all sets of flasks without the generation of NO_3-N. It can be postulated that the PEI binds the negatively charged NO_2^--N molecules to the bead surface leading to its disappearance. Besides inhibiting nitrification, these results lead to the realization that PEI was not a good immobilizing agent for nitrifiers.

4.3.3 Evaluation of PEI treated beads (PE) on the larvae of *Macrobrachium rosenbergii*

This experiment was conducted to assess the toxicity of PEI treated on prawn larvae and the results obtained are summarized in Table 4.3. Mortality of larvae during the experimental period was registered while in the experiment setup with untreated beads, the larvae remained stable. The observation points to the possible negative impact of PEI in larval rearing system.

4.3.4 Nitrifying potential of NIOPCU 1 suspended in growth medium

This experiment was conducted to clarify the point that the nitrifying consortium while suspended in growth medium could perform nitrification. As this information was essential to have a fresh look into the process of immobilization of nitrifiers onto the bead surface in the context that PEI has proved to be not suitable for affecting attachment of cells on the plastic beads. As summarized in the Table, NO_3-N generated was almost equal to the NO_2^--N consumed demonstrating nitrification by the freely suspended cells.

4.3.5 The influence of plastic beads (PEI) on the activity of free cells of nitrifiers

As summarized in Table 4, the presence of plastic beads did not alter in any way the nitrification of free cells in the growth medium suggesting that the appropriate and alternative method of immobilization can be worked out with out any loss of nitrifying activity.

4.3.6 Three hour immobilization of nitrifiers.

The extent of immobilization, which could be attained in a period of three hours as evidenced by the nitrification recorded, is summarized in Table 4.6. During the initial period of three hours nitrification could be recorded, as there was a build up of NO_3-N. Subsequently the beads were transferred to fresh medium no nitrification could be attained indicating that the attachment of cells to the beads has not taken place. This suggests a change in immobilization protocol.

4.3.7 Efficacy of saline as an immobilization fluid.

In this experiment the medium according to Watson (1965) was replaced with a 2 % saline and the extent of nitrification assessed. In this instance also no nitrification could be observed with the beads exposed to cells for three hours in saline. (Table 4.7).

4.3.8 Extent of attachment of cells during the three hour immobilization.

After three hour immobilization period the beads were washed and the nitrification potential of the washed beads and wash water was examined and summarized in Table 4.8. As well evident the wash water registered copious nitrification during a 48 hour period of incubation suggesting that the attachment of cells was reversible. Washed beads did not exhibit any nitrification at all.

4.3.9 Minimum time required for irreversible attachment of nitrifiers on beads

In this experiment plastic beads were exposed to a period of 72 hours with the assessment of attachment of three hours, 24, 48 and 72 hours. As summarized in Table 4.9 adsorption of cells could be evident from 48 hours onwards and 72 hour period it was considerably higher. This observation leads to the fixation of

72 hours as the minimum time period to be given for affecting adsorption of nitrifiers on beads.

4.3.10 Response of nitrifying consortium to different kinds of plastic beads moulded

This response was measured in terms of nitrification, which could be observed in the presence of beads. As summarized in table 4.10 generation of NO_3-N for a period of 72 hours in presence of beads was comparatively higher in the case of five types of beads such as HA, PD, PE, PF, and PG with that of control (without beads) in the presence of beads such as LB, PC and AH nitrification observed was slightly lesser compared to that of control. In any case no toxicity could be observed with any type of bead even though there was a difference in the extent of nitrification attained. These results lead to conceptualization of screening programme of all the eight kinds of plastic beads against each consortium to select the most appropriate one.

4.3.11 Selection of appropriate support material for each consortium.

Selection of appropriate support material for each consortium was made based on the following criteria.

1. Percentage of consumption of NH_4 + -N / NO_2 ˙ - N and production of NO_2^- - N / NO_3 – N during the immobilization phase and also with the beads having immobilized cells on the surface transferred to fresh medium
2. Cost of the type of plastic used
3. Easiness to mould into beads

Results of the experiments conducted are summarized in Table 4.11, 4.12, 4.13, and 4.14. Accordingly for AMOPCU 1 and AMONPCU 1, the plastic coded

as PE was selected as the most suitable one and for NIOPCU 1 and NIONPCU 1a, the plastic coded as LB was selected as the most ideal one.

4.3.12 Appropriate support materials for the immobilization of nitrifying consortium in the development of bioreactors

Significant observations which could be made through the series of experiments conducted above are: Polyethylene imine, an immobilization adsorption enhancer is toxic to nitrifying consortia developed here and it inhibits nitrification by a process not well known. This compound is to some extent lethal to prawn larvae. For the effective adsorption of nitrifying consortia on the surface of plastic beads it requires a period not less than 3 days and that also a simple agitation of the beads in the culture suspension at the optimum growth conditions would be enough with out the addition of any enhancer. This property of irreversible attachment of plastic surface could be seen very much with consortium, which was maintained after harvesting from the fermentor without centrifugation and washing. The reason for the ready attachment of the consortia can be ascribed to the undisturbed glycocalyx within which the cells are embedded and protected. In other words this is a reproduction of the natural process of bacterial adhesion on the solid substratum and to facilitate this consortium has to be maintained, undisturbed and applied at the optimum growth conditions. It is suspected that the cell free culture broth at the point of stationary phase contain an unknown compound, polymeric in nature, which enhances the natural adsorption of consortia to the plastic surfaces. This was experienced in one of the experiments when the cell free culture fluid was filtered through Sietz filter of 0.45μ porosity where it was not passing through!

The type of plastic selected finally for ammonia oxidizers and nitrite oxidizers such as the ones coded as PE and LB are easy to be moulded, easily available in the local market, comparatively less expensive also.

The scientific soundness of using plastic as a basic material as the support for nitrifiers has been justified by several workers. Sousa *et al.*, 1997 observed that nitrifying bacteria grow very slowly and bacterial washout from the reactor can be prevented by their immobilization from the fixed film on the biofilm reactors. The early stage of bacterial admission can be directed by Van der Waals forces of attraction and electrostatic forces of repulsion as formulated by DLVO theory (Oliveina, 1992)

According to the thermodynamic model, adhesion is favoured when the surface energy of the associated solids is lower than that of liquid medium. Generally plastics such as HD, PS, HDPE, PP, PVC, PMMA are used as the substrate. Both ammonia oxidizing and nitrite oxidizing bacteria exhibited negative surface charge during growth and the plastic support material also are negatively charged. This suggests that as per Van Loosdrecht *et al.*, (1990) surface hydrophobicity plays a dominant role because of Van der Waals force of attraction which increases with the surface hydrophobicity. Adhesion would be favoured if the surface of the support material is rubbed and carved with concentric lines in order to increase the roughness.

The plastic beads designed and moulded here have a surface area of > 0.785 cm^2. To increase the roughness and thereby to enhance adhesion, the surface is sparked. One of the outstanding achievements of the immobilization technology developed here is that, it is an extremely simple process, which does not involve the addition of any adsorption enhancers. Instead he optimum growth conditions are provided and the consortia are applied as such without any alteration or damage to the glycocalyx.

4.3.13 Designing, fabrication, activation of *in situ* stringed bed suspended reactor (SBSBR)

The stringed bed suspended bioreactors designed and fabricated are of standard size meant for employing in larval rearing systems during the culture operations. It was designed in such a way that if larvae enter the reactor it can easily comes out without getting mutilated. It is the case with the live food organisms. The cartridges are easy to be pulled out, washed and replaced. The kinetics of the reactor during activation and development are summarized with respect to each consortium in Table 4.15, 4.16, 4.17 and 4.18. The duration required for each consortium varies and in all cases end point was fixed as the disappearance of cells from suspension. In all cases nitrification was recorded at this stage and after draining away the fluid and recharging with fresh medium also nitrification went on unhampered, indicating that irreversible adsorption of the consortium has taken place. Both the stages of activation were done in an activation mode under optimum growth conditions. Subsequently the reactors were removed from the activation mode and deployed in seawater of 30 ppt / 15 ppt as per the requirements and as per the data presented in such situations, also nitrification went on uninterrupted. The sudden change of the reactor from the activation mode where the growth conditions were kept at optimum to the natural mode wherein other than the salinity, no other factors has been regulated did not inhibit or retarded nitrification. This could be ascribed to the property of biofilm reactors where the cells were protected in a glycocalyx and once a firm biofilm was formed minor variations in the environmental conditions did not alter the nitrification kinetics.

4.3.14 Field trial of *in situ* SBSBR in Penaeid hatchery

The result of a field trial of the in situ SBSBR in penaeid hatchery is summarized in Fig. 4.13, 4.14, 4.15 and 4.16. On deployment of the reactors, nitrification could be found to have taken place in the system with the bioreactor,

as the nitrite content in the water was found to be increasing with a decreasing NH_4^+-N content. During this phase NO_3^--N was not detectable, as the diatoms and the microalgae might have consumed it as the nitrogen source. Meanwhile in the control tank, production of nitrate-nitrogen was very limited even though an ammonia content of around 2.5 ppm was noticed at one instance indicating that nitrification was not taking place in this system. These two situations could be correlated with the survival and metamorphosis of the larvae. In the tanks with the reactor, more larvae could be enumerated everyday whereas, a sharp decline was observed in the control tank (Table 4.19). It has to be emphasized that during the entire rearing period no water exchange was given. Instead the quantity of water was increased from 600 to 900 L over a period of 17 days.

After the completion of the field trial the reactors were retrieved and transported to the lab with the 'water in' by keeping in a polythene bag in the laboratory the extent of activity of both the reactors were tested as described above and was found to be considerably enhanced (Table 4.20).

On testing at field level the reactor was found to be very much user friendly as the only job the hatchery personnel has to do was to connect the air lift pump to any of the existing aeration tubing and to regulate the air flow at the rate of 1 L min^{-1}. Rest all process are to be done by the manufacturer at the production level on commercialization of the technology.

In aquaculture systems attached growth approach to nurturing nitrifier growth has been recommended in lieu of a suspended growth strategy. Stationary plastic packing material (nested sheets, random beads, on saddles etc) represents an attractive media form, low in weight, high in specific surface area and chemically inert (Alleman and Preston, 1991). The type of plastic used in the development of reactors here follow the above qualities required to be used in the aquatic system. Alleman and Preston, (1991) characterized nitrifying biofilms to

have brown to orange brown colour and smell, earthy and musty. Interestingly the biofilms develop in the SBSBR had the above characteristic odour and colour.

A survey of literature revealed that the *in situ* stringed bed suspended bioreactor developed here is unique in its design, fabrication and mode of application.

[**Note** : The design has been recommended by Department of Biotechnology, Government of India for granting an Indian patent.]

4.3.15 Designing, fabrication and activation of *ex situ* packed bed rector

Activation kinetics of ex situ PBBR designed and fabricated for penaeid and non-penaeid hatchery systems are summarized in Table 4.21 and 4.22. in both the cases nitrification and disappearance of biomass from the medium in suspension could be noticed within 3 days. After drawing the fluid and on adding fresh seawater (30ppt in the case of reactors with AMONPCU 1 and NIOPCU 1 and 15ppt in the case of reactors with AMONPCU 1 and NIONPCU 1a), nitrification went on unhampered without any lag. This again indicated that once a biofilm is formed it becomes irreversible and stable enough to resist minor variations in the water quality parameters which normally happens in a hatchery system. On confirming activation of the reactors, water from the overhead tank can be drawn down to the reactors and an appropriate hydraulic retention time can be maintained to achieve nitrification.

The reactors designed and fabricated above are the standard types and the total number of such reactors required for each hatchery have to be decided upon based on the volume of water used per season. The reactors developed will enable

one to make the hatchery system closed for atleast a season and water can be reused and recirculated.

The configuration of the package of the PBBR to be offered there, is gravitational flow of water from the overhead tank A to tank C and successively to the reactors and finally to the collecting tank K. energy needed for the operation is only to pump water either spent water or fresh seawater after disinfection and salinity adjustment to the overhead tank. If the nitrification does not get completed alone it would be necessary to recirculate water through the treatment system over and again. But this can be effectively avoided by increasing units of nitrifying consortium used for the activation of the reactors and by regulating the hydraulic retention time.

In the bench scale bioreactor configuration described here the tanks A,C and K are of uniform size with 100L capacity made of fiberglass. The overhead tank is fixed on a metallic stand above tank C with the bottom outlet touching upon the water surface through a valve which can be regulated. The tank C serves as an aeration basin where degradation of organics can take place, provided the water is adequately aerated. One of the specialities of the design is that different types of filters for the removal of particulate matter, UV disinfection gadgets etc. can be connected online with the bioreactors as per the requirements without any modification of the system.

The *ex situ* PBBR has 9 aeration cells through which air can be passed through from 9 to 27 L min^{-1} indicating that there is a wide flexibility in the overall capacity of the system. It means that if the contents of NH_4^+-N and NO_2^--N in the water to be nitrified are more, it would be just sufficient to increase the quantity of air passed through the airlift pumps. This will enhance the contact time of water with the nitrifiers immobilized on beads.

Another speciality of the design is that the system which is used for penaeid prawn can be connected very easily for non-penaeid by just replacing the reactors connected in between the tanks with the ones meant for non-penaeid systems having the nitrifiers such as AMONPCU 1 and NIONPCU 1a immobilized on respective beads.

[**Note** : The design has been recommended by Department of Biotechnology, Government of India for granting an Indian patent.]

Tschui *et al.*, (1994) made extensive studies with biological filters using PS beads and ascertained that nitrification rates increased with increased water velocities along with increased airflow. Air velocity tests in the lab scale units revealed a gradual increase of the biological activity upto air ratio >30m/h. The higher activity at higher water and air velocities were basically explained by Tschui et al., (1994) by way of stronger turbulence in the filter, thereby the laminar boundary layer of the biofilm decreases and the mass flow increases by improved diffusion as the oxygen concentration in the system increases due to increased airflow. Moreover, the stronger turbulence may reduce hydraulic channeling and therefore improve advective transport in the granular media, leading to better utilization of the total attached biomass and to increase volumetric nitrification rates. In such situations the specific surface area of the filter bed increases and thereby small reactor volumes and compact plants become possible. The *ex situ* packed bed bioreactor developed here permits two directional flow of water, one horizontal and the other vertical. Both the flow rates can be regulated within a range increasing the efficiency of the biofilm formed.

4.4 Conclusion

Two types of reactors such as in situ stringed bed suspended bioreactor (SBSBR) meant for applying in larval rearing tanks during the larval rearing periods and *ex situ* packed bed reactor for nitrifying fresh seawater and spent water have been developed. The reactors meant for penaeid larval rearing system have to be activated with AMOPCU 1 and NIOPCU 1 and the ones meant for non-penaeid larval rearing system have to be activated with AMONPCU 1 and NIONPCU 1a. Activation of the reactors is to be done under optimum growth conditions of the consortium used. A field level evaluation of the *in situ* SBSBR conducted revealed that the reactor can be made use of effectively in nitrifying hatchery water and thereby the larval survival could be enhanced considerably. An effective implementation of the technology can make the penaeid and non-penaeid hatchery systems partially closed.

Table 4.1 Effect of polyethylene imine treatment of plastic beads on adsorption of the nitrifying consortium NIOPCU 1.

	Initial NO_2^- -N µg mL^{-1}	Final NO_2^- -N µg mL^{-1}	NO_2^- -N consumed µg mL^{-1}	Initial NO_3 -N µg mL^{-1}	Final NO_3 -N µg mL^{-1}	NO_3^-N produced µg mL^{-1}
Stage 1 Immobilization period (24 hours)						
Medium + Cells + Beads without PEI treatment	11.58	8.05	3.53	13.44	20.42	6.99
Medium + Cells + Beads with PEI treatment	11.30	5.23	6.07	0	0	0
Stage 2 24 hours after immobilization						
Medium alone without cells	12.21	12.20	0.01	0	0	0
Medium + Cells + Beads treated with PEI	12.33	10.84	1.49	0	0	0
Medium + Cells + Beads treated with PEI.	12.21	11.55	0.66	0	0	0

Table 4.2 Inhibition of nitrification (NIOPCU 1) in the presence of polyethylene imine

PEI concentration in percentage	Control(C) / Test (T)	NO_2^--N ($\mu g\ mL^{-1}$)			NO_3-N ($\mu g\ mL^{-1}$)		
		Initial	Final	Consumed	Initial	Final	Produced
0%	C	10.87	10.87	0	0	0	0
0%	T	11. 57	9.46	2.09	0	2.14	2.14
0.01%	C	12.27	11.01	1.26	0	0	0
0.01%	T	11.87	8.11	3.76	0	1.48	1.48
0.05%	C	11.50	11.78	0	0	0	0
0.05%	T	12.99	8.60	4.39	0	0	0
0.075%	C	11.55	12.27	0	0	0	0
0.075%	T	11.93	7.34	4.59	0	0	0
0.1%	C	12.10	12.10	0	0	0	0
0.1%	T	13.10	7.43	5.67	0	0	0
0.15%	C	12.67	12.68	0	0	0	0
0.15%	T	12.39	7.63	4.76	0	0	0
0.2%	C	12.90	12.90	0	0	0	0
0.2%	T	14.19	9.26	4.93	0	0	0

Note: C: Control (without inoculum)
T: Test (with inoculum.)

Table 4.3 Evaluation of the toxicity of polyethylene imine treated beads on the larvae of *Macrobrachium rosenbergii*

Type of Bead	Initial number of larvae	Number of larvae after 24 hours	Number of larvae after 48 hours.
Untreated beads	31	31	31
Treated beads	30	30	28
Without beads	35	35	35

Table 4.4 Nitrifying potential of the nitrite oxidising consortia NIOPCU 1 on suspending in growth medium.

Experimental set up	NO_2^- -N consumption ($\mu g\ mL^{-1}$)			NO_3 -N production ($\mu g\ mL^{-1}$)		
	Initial	Final	Consumed	Initial	Final	Produced
Medium alone	11.53	11.53	0	0	0	0
Medium along with consortium	10.81	10.06	0.75	0	0.86	0.86

Table 4.5 Influence of plastic beads on the activity of free cells of nitrifiers (NIOPCU I)

Experimental set up	NO_2^- -N consumption ($\mu g\ mL^{-1}$)			NO_3 -N production ($\mu g\ mL^{-1}$)		
	Initial	Final	Consumed	Initial	Final	Produced
Medium along with consortium	13.75	11.12	2.63	11.35	11.89	0.54
Medium along with consortium and plastic beads	13.87	11.44	2.44	11.34	11.90	0.56

Table 4.6 Extent of immobilization achieved by exposing plastic beads to the consortium for a duration of three hours.

Stages	NO_2^- -N consumed ($\mu g\ mL^{-1}$)			NO_3 -N produced ($\mu g\ mL^{-1}$)		
	Initial	Final	Consumed	Initial	Final	Produced
Stage 1 Immobilization period						
a. Control (Medium + Beads without inoculum)	11.35	11.29	0.06	0	0	0
b. Test (Medium + Beads with Inoculum)	11.58	8.06	3.53	13.44	20.42	6.98
Stage 2 24 hours after immobilization						
a. Control (Medium + Beads without inoculum)	11.53	11.70	0	0	0	0
b. Test (Medium + Beads with Inoculum	12.33	10.84	1. 49	0	0	0

ble 4.7 Efficacy of saline as an alternative immobilization fluid

ups	NO_2^- -N consumed ($\mu g\ mL^{-1}$)			NO_3 -N produced ($\mu g\ mL^{-1}$)		
	Initial	Final	Consumed	Initial	Final	Produced
up 1						
ring 3 hours mobilization od						
Saline + Consortium	11.95	16.00	0	0	0	0
Growth Medium + Consortium	11.83	8.06	3.77	13.44	20.42	6.98
up 2						
Hours after mobilization						
Saline + Consortium	11.96	10.92	1.04	0	0	0
Growth Medium + Consortium	12.33	10.34	1.49	0	0	0

4.8 Extent of attachment of cells (NIOPCU I) during the three hours immobilization

	NO_2^- -N consumed (μg mL^{-1})				NO_3 -N produced (μg mL^{-1})			
	Initial	Final		Consumed	Initial	Final		Produced
1 3 hours obilization od ium + sortium + ds	11.58	8.06		3.52	13.44	20.42		6.98
2 hours after obilization	Initial	24 hr.	48 hr.	Consumed	Initial	24 hr.	48 hr.	Produced
Control (Wash water)	10.15	6.45	0.06	10.09	0	6.53	12.16	12.16
Test (Medium + Beads)	12.33	10.84	10.35	1.98	0	0	0	0
Beads without washing	11.97	10.06	8.05	3.92	0	0.95	1.50	1.50

Table 4.9 Minimum time required for irreversible attachment of nitrifiers (NIOPCU I) on beads

Stages	NO_2^--N consumed ($\mu g\ mL^{-1}$)				NO_3-N produced ($\mu g\ mL^{-1}$)			
	3 hrs.	24 hrs.	48 hrs.	72 hrs.	3 hrs.	24 hrs.	48 hrs.	72 hrs.
Stage 1 During the immobilization phase	3.52	9.63	10.53	12.17	6.98	4.28	8.50	13.62
Stage 2 Beads in fresh medium after every time period of immobilization	1.49	1.73	1.80	1.85	0	0	1.50	2.13

Table 4.10 Response of the nitrifying consortium to different kinds of plastic beads during a period of 72 hours incubation.

Experimental sets	NO_2^- -N consumed ($\mu g\ mL^{-1}$)			NO_3 -N produced ($\mu g\ mL^{-1}$)		
	Initial Quantity	Final Quantity	Quantity Consumed	Initial Quantity	Final Quantity	Quantity Produced
1. Control (Cells + Medium)	9.61	0.39	9.22	60	14.07	9.47
2. HA*	11.78	0.03	11.75	0	11.37	11.37
3. LB	11.12	2.85	8.27	0	8.14	8.14
4. PC	12.07	0.26	11.81	0	4.32	4.32
5. PD	11.24	0.03	11.21	0	12.35	12.35
6. PE	10.69	0.13	10.56	0	12.23	12.23
7. PF	11.21	0	11.21	0	11.67	11.67
8. PG	11.15	0	11.15	0	12.56	12.76
9. AH	12.16	5.03	7.13	0	6.19	6.19

* Code given for each type of plastic used for moulding the beads. Actual names are masked at the request of Department of Biotechnology, the funding agency.

Table 4.11 Selection of appropriate support material for AMOPCU I

Sl no	Types of plastic beads used	Nitrification during immobilisation phase		Nitrification with the consortium immobilised on beads		Cost Rs. per kg	Easiness to mould
		% NH_4^+-N consumed	% NO_2^--N produced	% NH_4^+-N consumed	% NO_2^--N produced		
1	HA	2.67±2.05	5.95 ±8.41	22.03±2.56	48.35 ±66.75	545	Very easy
2	LB	16.96±13.06	41.72±35.42	95.93±5.75	94.01±21.19	560	Easy
3	PC	89.90 ± 8.90	3.80 ±2.63	100± 0	0.07± 0.10	575	Difficult
4	PD	25.98 ±7.89	49.21±1208	99.06±1.33	86.56±20.76	525	Very easy
5	PE	22.11±19.28	75.92±21.66	100± 0	91.35 ±9.25	535	Easy
6	PF	25.61±11.70	53.89±33.07	86.23±11.98	115.44±15.51	700	Difficult
7	PG	34.47±3.22	42.96±6.57	94.29±8.07	102.54±4.67	600	Difficult
8	AH	28.7±20.33	13.91±9.85	90.12±12.01	111.22±47.56	650	Easy

Table 4. 12 Selection of appropriate support material for NIOPCU I

Sl no	Types of plastic beads used	Nitrification during immobilisation phase		Nitrification with the consortium immobilised on beads		Cost Rs. per kg	Easiness to mould
		% NO_2^--N consumed	% NO_3-N produced	% NO_2^--N consumed	% NO_3-N produced		
1	HA	99.34±0.78	129.16±13.05	35.75±45.47	22.55±31.89	545	Very Easy
2	LB	98.55±1.19	120.08±4.68	100±0	69.93±2.01	560	Easy
3	PC	99.07±0.19	47.296±13.88	99.82±0.25	1.367±1.56	575	Difficult
4	PD	99.04±0.67	119.83±28.12	91.53±11.59	64.01±18.42	525	Very Easy
5	PE	99.46±0.31	111.39±0.44	99.83±0.25	63.04±15.24	535	Easy
6	PF	99.22±0.26	109.44±7.25	100±0	75.00±4.73	700	Difficult
7	PG	99.098±0.36	113.77±4.78	99.82±0.25	90.27±7.25	600	Difficult
8	AH	99.330±1.90	109.66±20.04	98.03±2.08	72.19±0.2	650	Easy

Table 4.13 Selection of appropriate support material for AMONPCU I

Sl No	Types of plastic beads used	Nitrification during immobilisation phase		Nitrification with the consortium immobilised on beads		Cost Rs. per kg	Easiness to mould
		% NH_4^+-N consumed	% NO_2^--N produced	% NH_4^+-N consumed	% NO_2^--N produced		
1	HA	96.36±2.07	90.56±16.34	92.34±13.24	100± 0	545	Easy
2	LB	100±0	100±0	88.94 ±15.06	100± 0	560	Difficult
3	PC	100±0	91.49±14.75	71.32± 18.98	88.55±19.14	575	Very easy
4	PD	98.76±0.93	100±0	99.84± 0.28	100± 0	525	Easy
5	PE	99.84±0.27	100±0	100 ±0	100± 0	535	Difficult
6	PF	97.96±3.54	100±0	98.76± 2.15	100± 0	700	Easy
7	PG	99.52±0.849	100±0	100± 0	100± 0	600	Difficult
8	AH	3.75±10.8	100±0	100±0	100± 0	650	Easy

Table 4.14 Selection of appropriate support material for NIOPCU I

	Type of plastic beads used	Nitrification during immobilisation phase		Nitrification with the consortium immobilised on beads		Cost Rs.per kg	Easiness to mould
		% NO_2^--N consumed	% NO_3-N produced	% NO_2^--N consumed	% NO_3-N produced		
	HA	77.62±6.54	71.92± 38.14	85.55 ±2.52	41.26 ±9.998	545	Very Easy
	LB	82.13 ±1.41	63.84± 5.59	83.05±2.08	49.31 ±9.77	560	Easy
	PC	81.98±1.56	24.29 ±11.57	84.33±3.22.	50.78± 8.01	575	Difficult
	PD	81.69±1.64	57.81 ±21.81	84.83 ±0.42	29.07± 18.24	525	Very Easy
	PE	80.41±0.71	68.2 ±8.73	85.16± 1.61	50.69± 10.32	535	Easy
	PF	78.26±3.52	40.22 ±30.55	84.5± 0.68	61.79± 2.19	700	Difficult
	PG	82.53±0.75	69.31± 8.84	84.35 ±0.41	73.67 ±7.76	600	Difficult
	AH	80.04±1.96	53.83 ±22.58	82.57 ±2.1	63.6± 12.77	650	Easy

ble 4.15 Activation kinetics of *in situ* stringed bed suspended bioreactor immobilized with MOPCU I.

...ation ...de	NH_4^+ -N consumption (μg mL^{-1})			NO_2^- -N production (μg mL^{-1})			% nitrifying biomass uptake (mg) on 3rd day
	Initial content	Content on 8th day	% consumption	Initial content	Content on 8th day	% production out of NH_4^+ -N consumed	
...ge 1							
...trol 1	8.106	8.124	Nil	0	0	0	-
...trol 2	8.02	7.998	Negligible	0	0	0	-
...s1	7.35	1.932	73.71	0	9.899	182.71	42.68
...s2	7.686	1.638	78.69	0	9.999	165.24	43.37
...s3	6.972	1.032	85.2	0	9.199	154.87	39.25
...s4	7.728	1.310	83.05	0	8.333	129.83	30.88
	Mean ± SD 81.162 ± 5.083			Mean ± SD 158.16 ± 22.11			Mean ± SD 39.045± 5.73
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% production of NH_4^+ -N consumed	
...ge 2							
...trol 1	8.064	9.112	Nil	0	0	0	N.A.
...trol 2	7.980	7.842	Nil	0	0	0	
...s1	8.526	5.512	35.35	0	3.216	106.702	
...s2	7.728	5.44	29.61	0	2.884	126.050	
...s3	7.140	5.46	23.53	0	3.146	187.260	
...s4	7.560	5.964	21.11	0	3.339	174.130	
	Mean ± SD 27.4 ± 6.39			Mean ± SD 148.54 ± 38.34			
	Initial content	Content on 4th day	% consumption	Initial content	Content on 3rd day	% production of NH_4^+ -N consumed	
...ge 3							
...trol 1	8.184	8.212	Nil	0	0	0	N.A.
...trol 2	7.998	7.868	Nil	0	0	0	
...s1	8.105	1.596	80.308	0	8.299	127.80	
...s2	8.031	2.058	74.374	0	8.399	140.63	
...s3	8.202	0.126	98.46	0	11.698	144.86	
...s4	8.481	0	100.0	0	10.632	125.37	
	Mean ± SD 88.29 ± 12.88			Mean ± SD 134.59 ± 9.61			

...: Not applicable
...e1: During activation in the activation mode
...e2: With change of fluid in the activation mode
...e3: On transporting to simulated hatchery system

Table 4.16 Activation kinetics of *in situ* stringed bed suspended bioreactor immobilized with NIOPCU I.

Activation mode	NO_2^--N consumption ($\mu g\ mL^{-1}$)			NO_3^--N production ($\mu g\ mL^{-1}$)			% nitrifying biomass uptake (mg) on 3rd day
	Initial content	Content on 5th day	% consumption	Initial content	Content on 8th day	% production out of NO_2^--N consumed	
Stage 1							
Control 1	12.998	12.468	Negligible	0	0	Nil	
Control 2	12.665	12.684	Negligible	0	0	Nil	
Test 1	12.123	1.333	89.00	0	8.933	82.79	100
Test 2	12.899	0.633	94.68	0	9.247	82.08	100
Test 3	11.632	0.549	95.28	0	9.365	84.49	100
Test 4	12.398	0.984	92.06	0	10.443	91.49	100
	Mean ± SD 92.76 ± 2.87			Mean ± SD 85.21 ± 4.31			Mean ± SD 100 ±0
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% production of NO_2^--N consumed	
Stage 2							
Control 1	12.83	14.298	Nil	0	0	Nil	
Control 2	13.05	14.336	Nil	0	0	Nil	
Test 1	13.332	0	100	0	22.07	165.5 5	N.A.
Test 2	12.165	0.467	96.16	0	18.498	158.13	
Test 3	11.665	0	100	0	20.337	194.33	
Test 4	11.366	0.324	97.15	0	19.364	175.38	
	Mean ± SD 98.33 ± 1.99			Mean ± SD 168.35 ± 8.11			
	Initial content	Content on 6th day	% consumption	Initial content	Content on 6rd day	% production of NO_2^--N consumed	
Stage 3							
Control 1	14.765	14.89	Nil	0	0	0	
Control 2	14.789	14.86	Nil	0	0	0	
Test 1	13.224	0	100	0	11.369	85.97	N.A.
Test 2	14.124	0.212	98.50	0	10.892	78.29	
Test 3	12.332	0	100	0	11.468	92.99	
Test 4	14.886	0	100	0	11.678	78.45	
	Mean ± SD 99.68 ± 0.75			Mean ± SD 83.93 ± 7.025			

N.A : Not applicable

Stage 1: During activation in the activation mode

Stage 2: With change of fluid in the activation mode

Stage 3: On transporting to simulated hatchery system

ble 4.17 Activation kinetics of *in situ* stringed bed suspended bioreactor immobilized with MONPCU 1

ivation de	NH_4^+ -N consumption ($\mu g\ mL^{-1}$)			NO_2^- -N production ($\mu g\ mL^{-1}$)			% nitrifying biomass uptake (mg) on 3rd day
	Initial content	Content on 6th day	% consumption	Initial content	Content on 6th day	% production out of NH_4^+ -N consumed	
ge 1							
ntrol 1	10.148	10.542	Nil	0	0	0	-
ntrol 2	10.77	13.566	Nil	0	0	0	-
st 1	10.342	0	100	0	11.386	110.09	85.25
st 2	10.484	0	100	0	14.595	139.22	81.54
st 3	10.862	0	100	0	11.096	102.16	90.48
st 4	10.392	0	100	0	11.728	112.86	90.41
	Mean ± SD 100.0±0			Mean ± SD 116.09 ± 16.08			Mean ± SD 86.92 ± 4.34
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% production of NH_4^+ -N consumed	
ge 2							
ntrol 1	10.178	10.988	Nil	0	0	0	
ntrol 2	10.162	10.344	Nil	0	0	0	
st 1	10.69	2.856	73.28	0	13.396	171.008	N.A.
st 2	10.68	2.016	81.13	0	14.963	172.67	
st 3	10.23	1.344	86.866	0	16.830	189.40	
st 4	10.456	1.150	89.00	0	18.729	201.26	
	Mean ± SD 82.549 ± 7.011			Mean ± SD 183.58 ± 14.42			
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% production of NH_4^+ -N consumed	
ge 3							
ntrol 1	10.906	10.894	Negligible	0	0	0	
ntrol 2	10.654	10.689	Nil	0	0	0	
st 1	10.738	0.924	91.39	0	11.732	119.55	
st 2	10.822	0.714	93.40	0	12.065	119.37	N.A.
st 3	10.780	0.924	91.43	0	11.265	114.30	
st 4	10.738	1.260	88.27	0	12.732	134.33	
	Mean ± SD 91.123 ±			Mean ± SD 121.888 ±			

A : Not applicable
ge 1: During activation in the activation mode
ge 2: With change of fluid in the activation mode
ge 3: On transporting to simulated hatchery system

:e4.18 Activation kinetics of *in situ* stringed bed suspended bioreactor immobilized with :NPCU 1.

:vation :te	NO_2^- -N consumption ($\mu g\ mL^{-1}$)			NO_3 -N production ($\mu g\ mL^{-1}$)			% immobilization of nitrifying biomass
	Initial content	Content on 6th day	% consumption	Initial content	Content on 6th day	% production out of NO_2^- -N consumed	
ge 1							
ntrol 1	8.733	8.683	Negligible	0	0	0	
ntrol 2	9.965	9.366	Negligible	0	0	0	
st 1	8.499	0	100	0	14.858	174.81	92.31
st 2	8.499	0	100	0	14.778	173.87	73.53
st 3	8.265	0.033	99.60	0	13.664	165.97	51.56
st 4	8.533	0.1667	98.05	0	14.284	170.75	72.46
	Mean ± SD 99.413 ± 0.927			Mean ± SD 171.35 ± 3.98			Mean ± SD 72.46 ± 16.65
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% production of NO_2^- -N consumed	
ge 2							
ntrol 1	10.132	10.566	Nil	0	0	0	
ntrol 2	10.132	9.299	Nil	0	0	0	
st 1	10.132	0	100	0	21.625	213.43	N.A
st 2	10.132	0	100	0	16.4813	162.66	
st 3	10.132	0.0333	99.67	0	12.3217	122.01	
st 4	10.132	0	100	0	17.995	177.59	
	Mean ± SD 99.918 ± 0.165			Mean ± SD 168.923 ± 37.84			
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% production of NO_2^- -N consumed	
ge 3							
ntrol 1	11.499	13.932	Nil	0	0	0	
ntrol 2	11.499	14.498	Nil	0	0	0	N.A
st 1	11.499	0	100	0	15.0	130.45	
st 2	11.499	0	100	0	14.48	125.88	
st 3	11.499	0.0667	99.42	0	14.38	125.78	
st 4	11.499	0.1999	98.26	0	14.57	128.95	
	Mean ± SD 99.42 ± 0.82			Mean ± SD 127.76 ± 2.32			

\A. :Not applicable
ge 1: During activation in the activation mode
ge 2: With change of fluid in the activation mode
ge 3: On transporting to simulated hatchery system

Table 4.19 Levels of NH_4-N, NO_2-N, NO_3-N, salinity, pH and the number of larvae survived through various stages in the larval rearing system with and without the *in situ* SBSBR

Without *in situ* SBSBR

Days	Larval Stage	NH_4 $\mu g\ mL^{-1}$	NO_2 $\mu g\ mL^{-1}$	NO_3 $\mu g\ mL^{-1}$	Salinity ‰	pH	No: of larvae (approx.)
1	Egg	0.924	0	0	33	8	
2	Nauplius	0.462	0.1333	0	37	8	84280
3	Nauplius	0.504	0	0.09554	36	8	84280
4	Nauplius	0	0	0.3344	36	8	61600
5	Zoea	0.084	0	0.7166	36	8	60000
6	Zoea	0.924	0.0333	0.04781	36	8	60000
7	Zoea	0.378	0	0.1433	36	8	50000
8	Mysis	0.42	0.2599	0.3399	36	8	50000
9	Mysis	0.798	0.0333	0	37	8	49920
10	Mysis	1.806	0	0.1991	35	7.5	49000
11	PL 1	1.26	0	0.1911	37	8	45000
12	PL 2	1.092	0	0.2389	32	8	40000
13	PL 3	1.261	0	0.09554	38	8	38840
14	PL 4	2.536	0.1999	0	35	8	37372
15	PL 5	1.512	0.06666	0	32	8	35720
16	PL 6	1.428	0.09999	0.1899	35	8	34378
17	PL 7	1.428	0.1999	0	37	8	33728

With the *insitu* SBSBR

Days	Larval Stage	NH_4 $\mu g\ mL^{-1}$	NO_2 $\mu g\ mL^{-1}$	NO_3 $\mu g\ mL^{-1}$	Salinity ‰	pH	No:of larvae (approx.)
1	Egg	0.21	0	0	35	8	
2	Nauplius	0.966	0.1999	0.3344	36	8	100940
3	Nauplius	1.47	0.0333	0.09559	35	8	93248
4	Nauplius	0.042	0.2999	0	35	8	93240
5	Zoea	0.084	0.0999	0.4777	35	8	93240
6	Zoea	0.462	0	0.0477	35	8	93230
7	Zoea	0.546	0	0	35	8	93200
8	Mysis	0.462	0	0.1433	35	8	93192
9	Mysis	0.4803	0.09999	0.04777	37	8	93176
10	Mysis	1.722	0.2999	0	35	8	92125
11	PL 1	1.659	0.09999	0.5255	36	8	91256
12	PL 2	1.029	0.5333	0	34	8	89913
13	PL 3	0.946	1.1666	0	36	8	89861
14	PL 4	1.218	2.4331	0	36	8	89729
15	PL 5	1.008	3.4329	0	38	7.5	88996
16	PL 6	0.252	4.4329	0	33	8	88960
17	PL 7	0	4.5329	0	36	8	87296

Table 4.20 Nitrifying potential of the *in situ* SBSBR before transporting to the hatchery for deployment and their activity on retrieval after 17 days of operation in the hatchery

Reactors	Before transportation		After retrieval	
	Substrate used per day ($\mu g\ mL^{-1}$)	Product formed per day ($\mu g\ mL^{-1}$)	Substrate used per day ($\mu g\ mL^{-1}$)	Product formed per day ($\mu g\ mL^{-1}$)
Ammonia oxidizing reactor 1	7.7955	7.1993	10.668	10.5
Ammonia oxidizing reactor 2	4.3608	4.0663	10.458	10.42
Nitrite oxidizing reactor 1	3.2545	3.22	7.4992	7.4
Nitrite oxidizing reactor 2	5.7327	3.344	7.9658	7.95

Table 4.21 Activation kinetics of *ex situ* packed bed bioreactor designed and fabricated for penaeid larval rearing system

Activation mode	NH_4^+ -N consumption ($\mu g\ mL^{-1}$)			NO_2^- -N production ($\mu g\ mL^{-1}$)			% adsorption of nitrifying biomass
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% consumption	
Reactor F1 (AMOPCU1) **Stage 1**	6.51	3.024	53.54	7.1993	15.565	239.98	100
Stage 2	4.452	0	100	0.0666	1.699	34.59	NA
Reactor F2 (NIOPCU 1) **Stage 1**	10.465	4.833	53.82	10.701	13.076	42.17	100
Stage 2	10.499	5.399	51.43	0	2.723	50.43	NA

NA. : Not applicable
Stage 1 : During activation in the activation mode
Stage 2 : With change of fluid in the activation mode

Table 4.22 Activation kinetics of *ex situ* packed bed bioreactor designed and fabricated for non-penaeid larval rearing system

Activation mode	NH_4^+ -N consumption ($\mu g\ mL^{-1}$)			NO_2^- -N production ($\mu g\ mL^{-1}$)			% adsorption of nitrifying biomass
	Initial content	Content on 3rd day	% consumption	Initial content	Content on 3rd day	% consumption	
Reactor F1 (AMONPCU 1) **Stage 1**	10.034	2.268	77.40	6.433	9.799	43.35	100
Stage 2	7.35	0	100	0.733	15.798	214.9	NA
Reactor F2 (NIONPCU 1a) **Stage 1**	9.699	6.033	54.99	0	5.3983	101.15	100
Stage 2	14.232	4.999	35.13	0	3.007	32.55	NA

NA. : Not applicable
Stage I : During activation in the activation mode
Stage 2 : With change of fluid in the activation mode

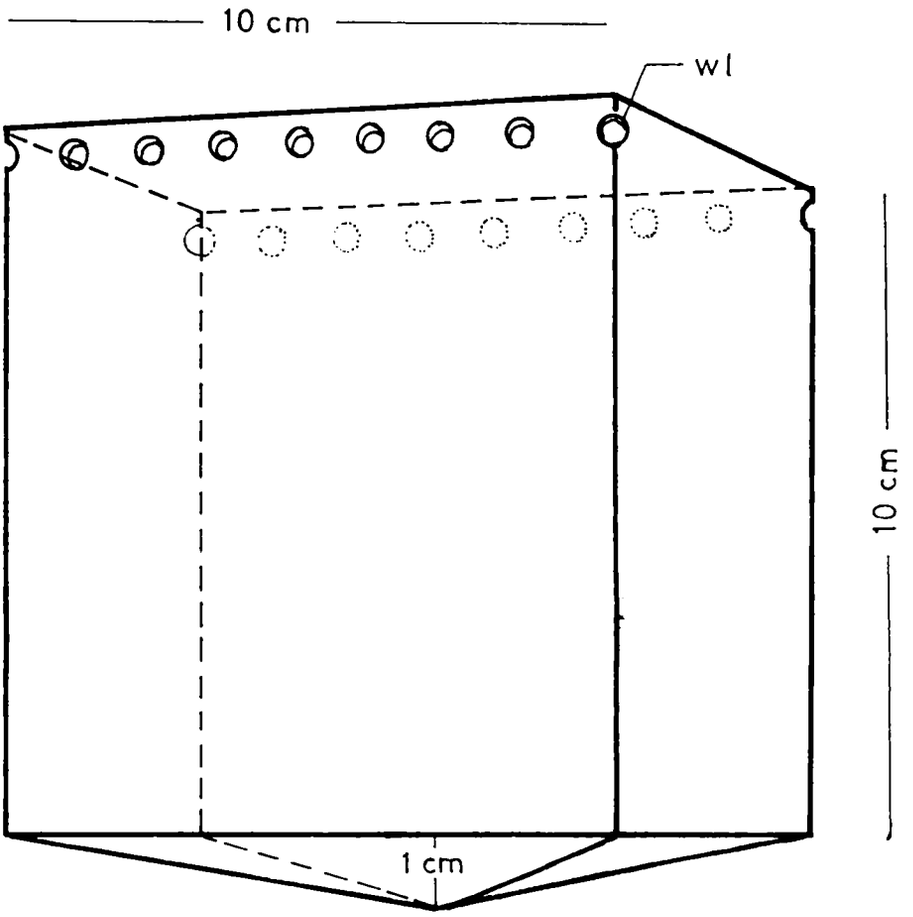


Fig 4.1 **Design of the shell of SBSBR**
W1 : Water inlet

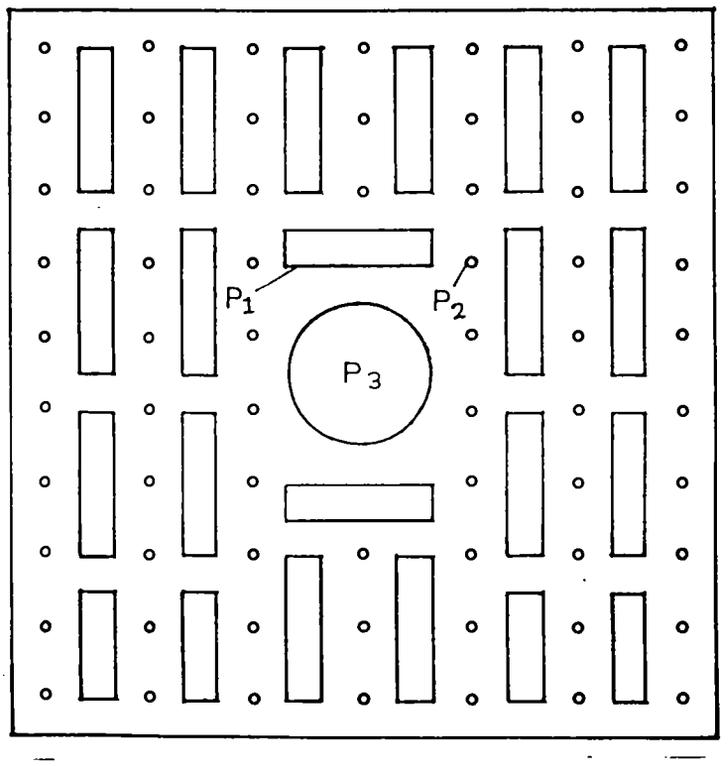


Fig 4.2 Design of the filter plate of the inner cartridge of SBSBR
P1: Slit through which water circulates, 5mm width and 1to 1.5 cm Length.
P2: Hole through which the thread is wound.
P3: Opening for the central air lift pump.

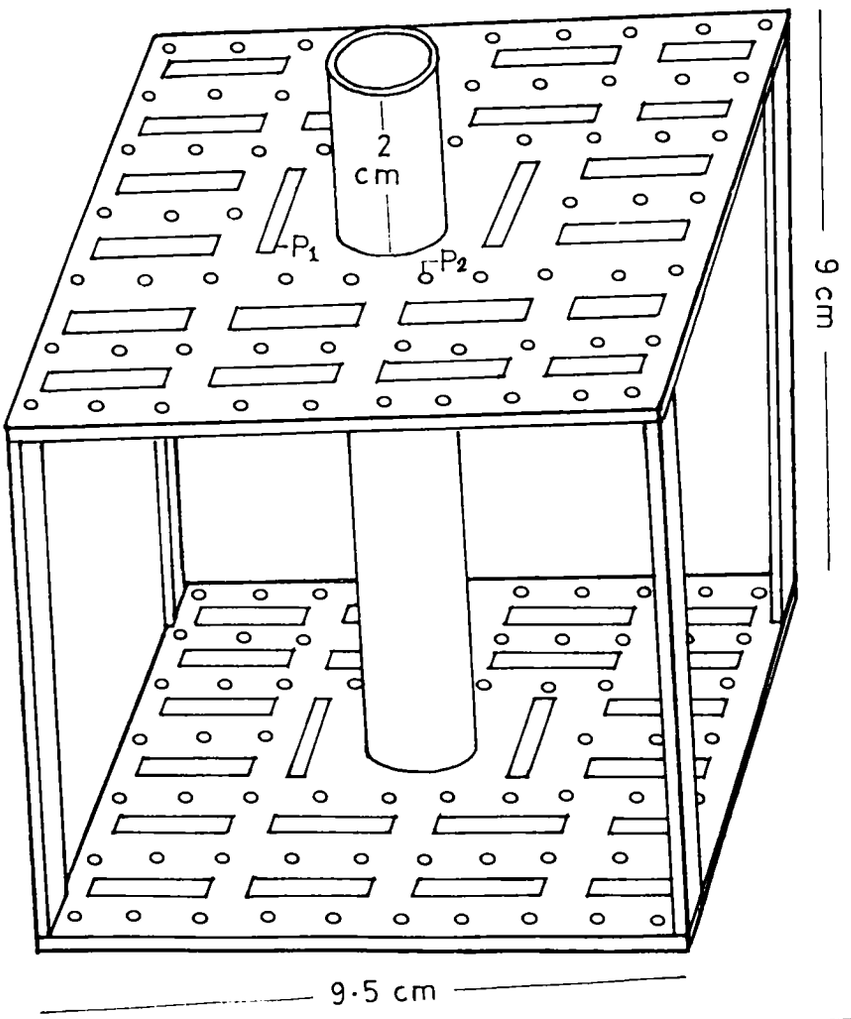


Fig 4.3 Design of the frame work for the inner cartridge of SBSBR
P1 : Slit through which water circulates
P2: Hole through which the thread is wound

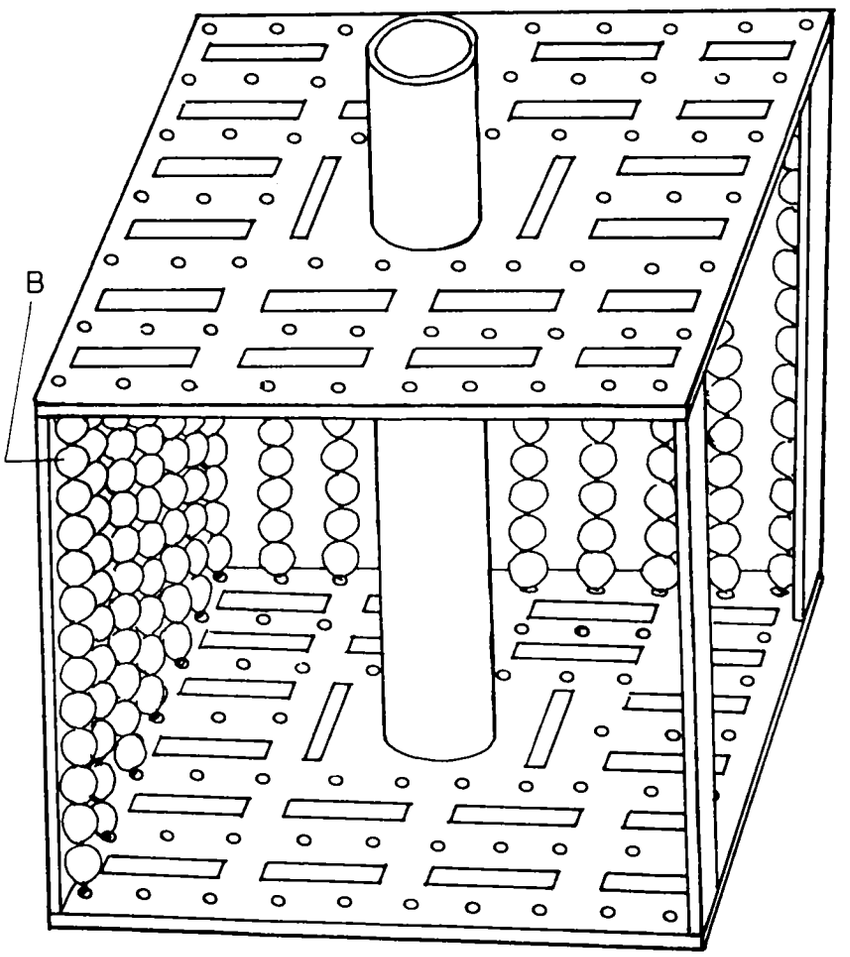


Fig 4.4 Design of the inner cartridge of SBSBR
B: Plastic beads on strings

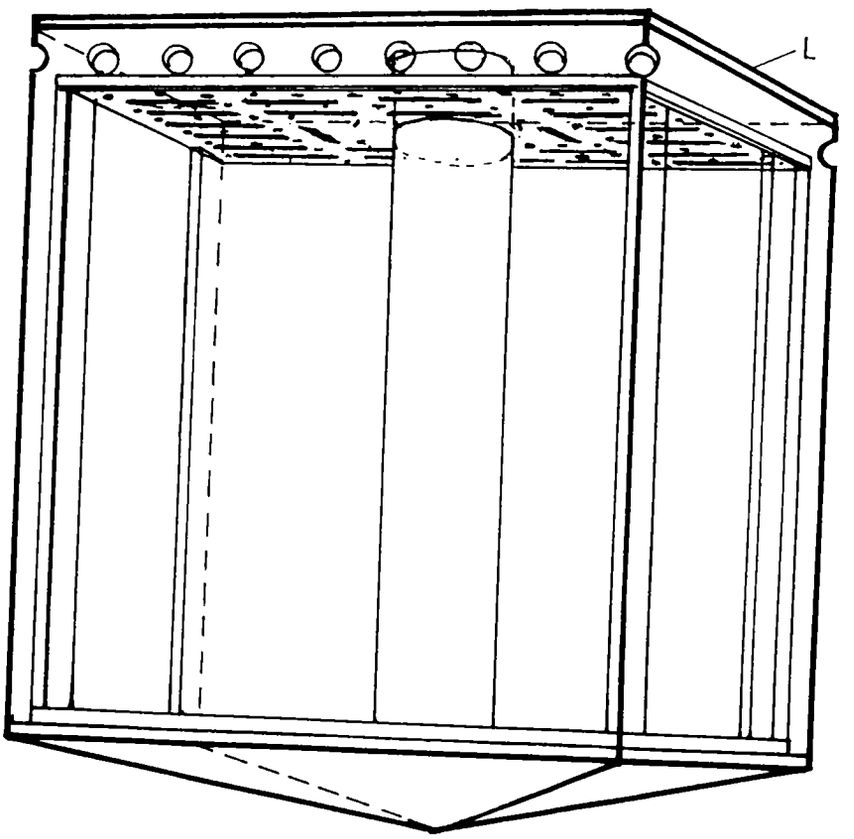
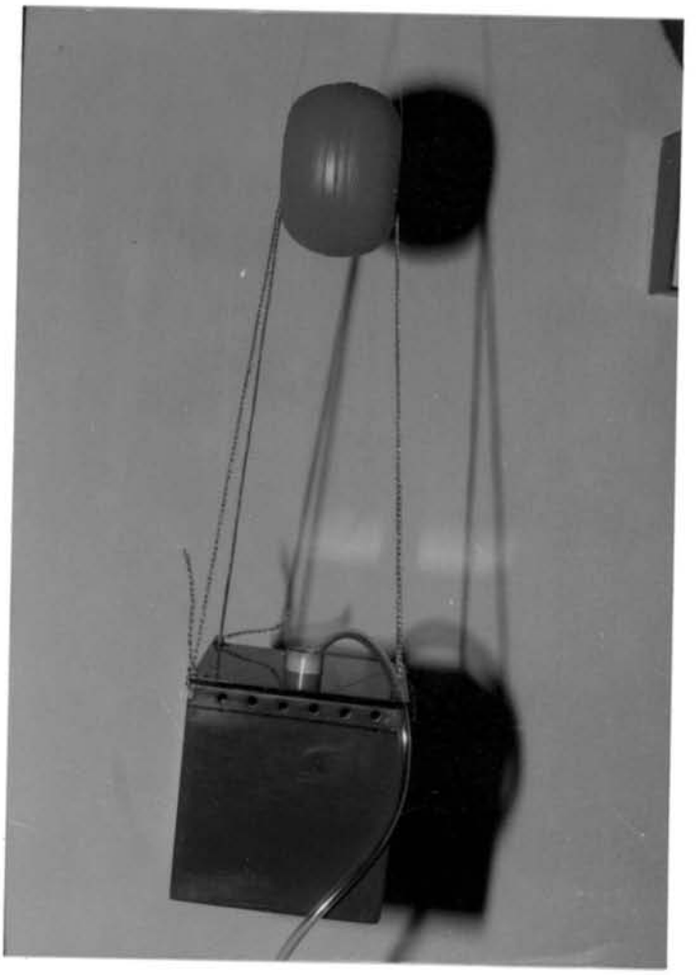


Fig 4.5 Design of SBSBR on assembly
L: Top lid of SBSBR

Fig 4.6 SBSBR after activation prepared for transportation along with float

Fig 4.7 SBSBR deployed in a penaeid larval rearing system



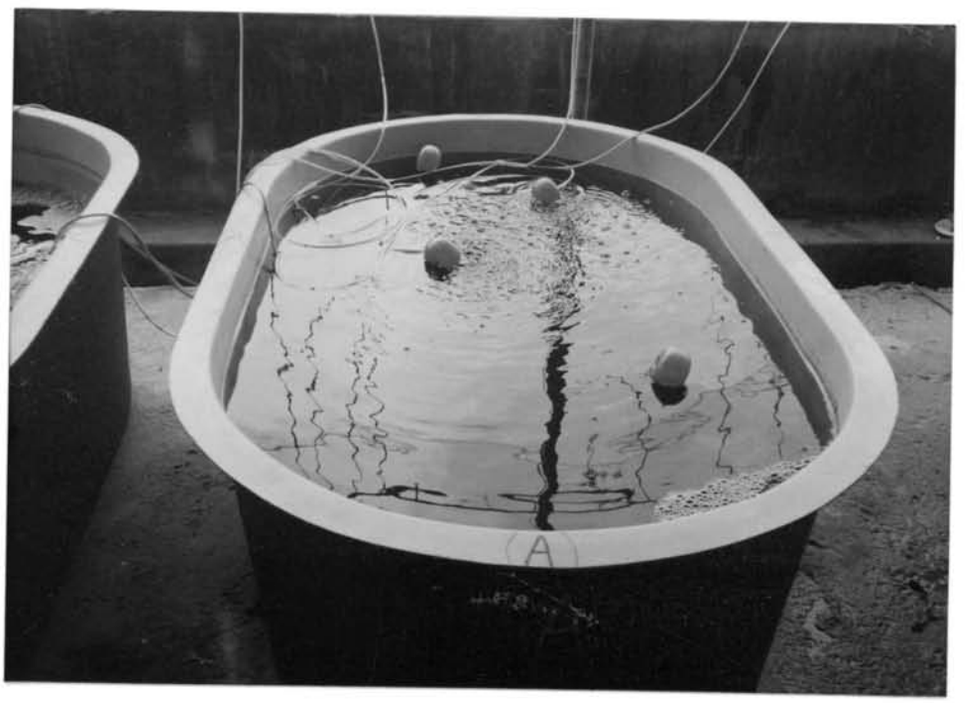
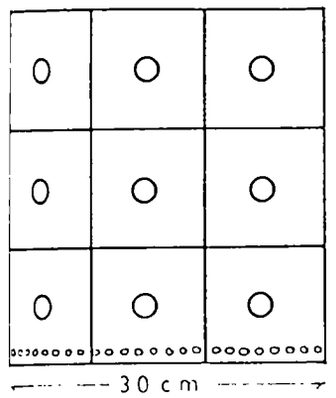
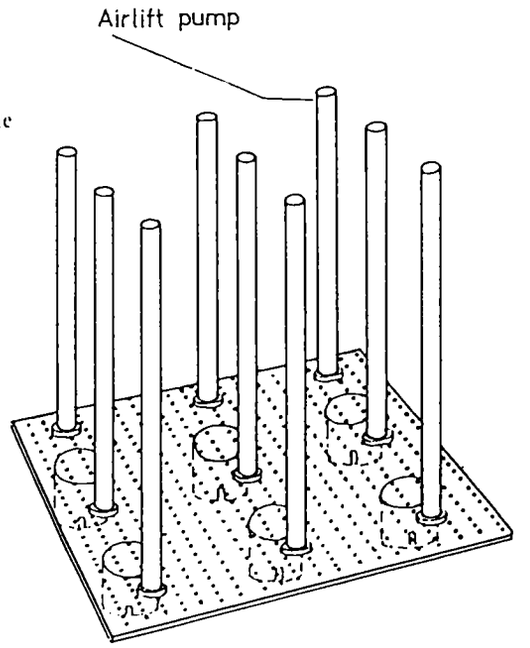
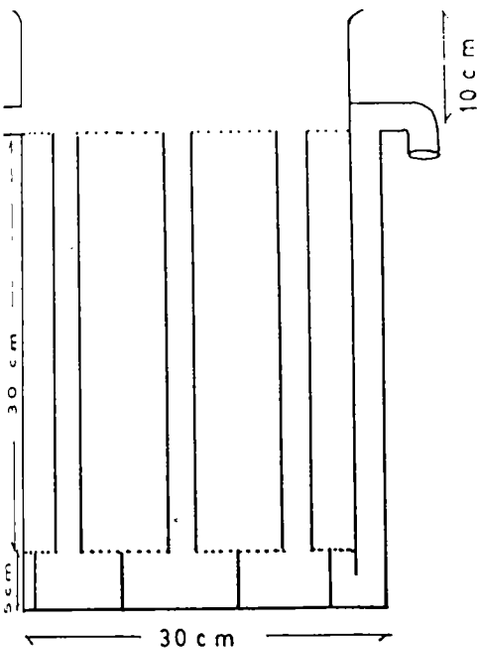

A

Fig. 4.8 Design of the air lift pump fixed on base plate forming 9 aeration cells.
Length: 29 cm, diameter: 2cm







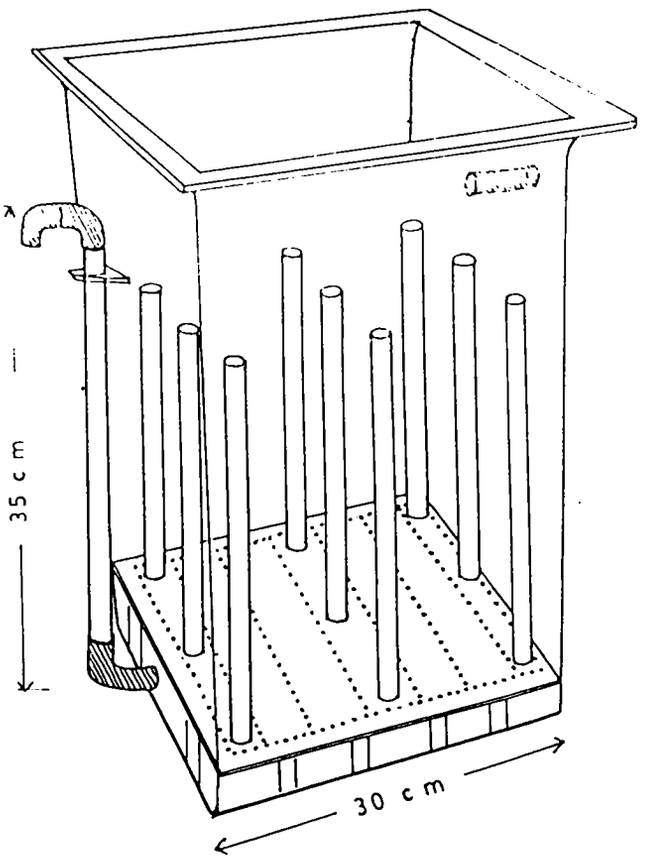
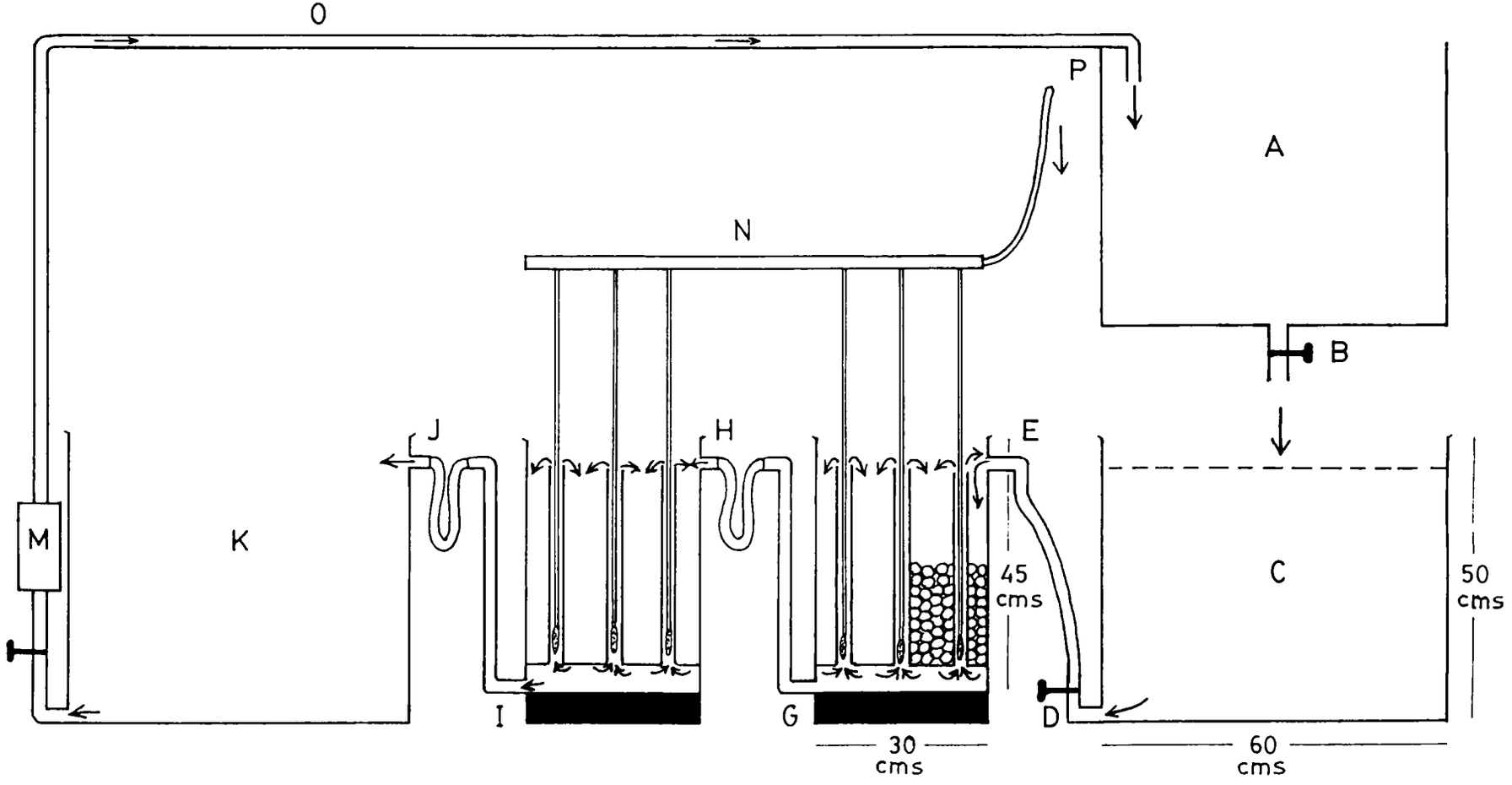


Fig 4.9 Design of the PBBR.

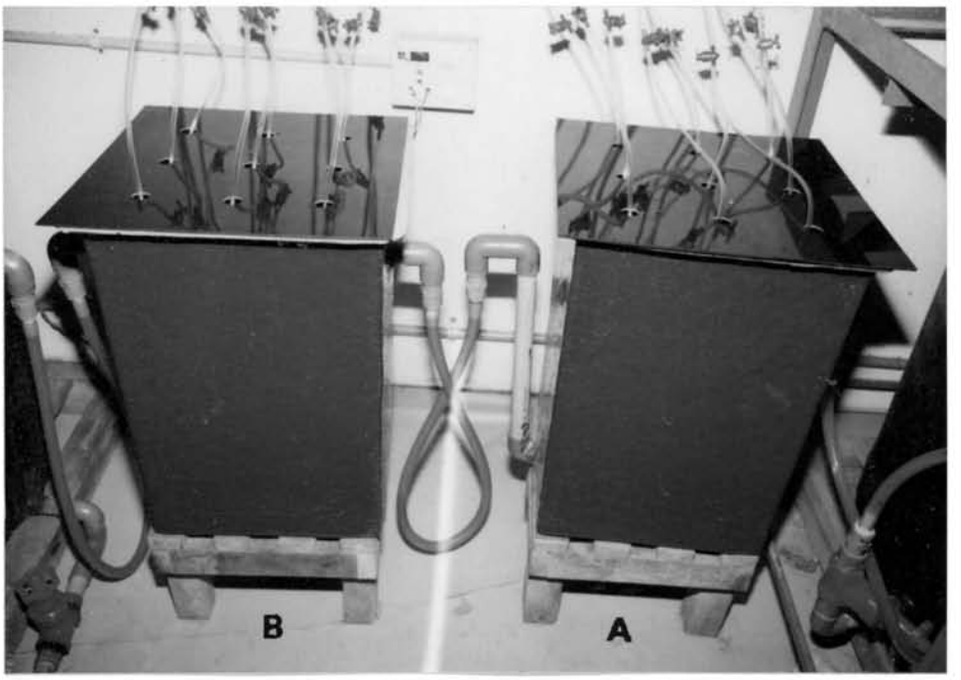
A. Overhead tank; B. Valve; C. Ground level aeration basin;
D. Outlet of C; E. Inlet of PBBR for ammonia oxidation;
F. Ammonia oxidizing bioreactor; G. Outlet of F; H. Inlet of nitrite oxidizing bioreactor; I. Outlet of nitrite oxidizing bioreactor; J. Inlet of collecting tank K; M. Magnetic coupled pump; O. Return pipe to overhead tank



F2 F1

Fig 4.11 PBBR fabricated in full assembly
A. Ammonia oxidizing reactor B. Nitrite oxidizing reactor

Fig 4.12 PBBR fabricated in full assembly
C. Ground level aeration basin D. Collecting tank.


B
A


E
F
G

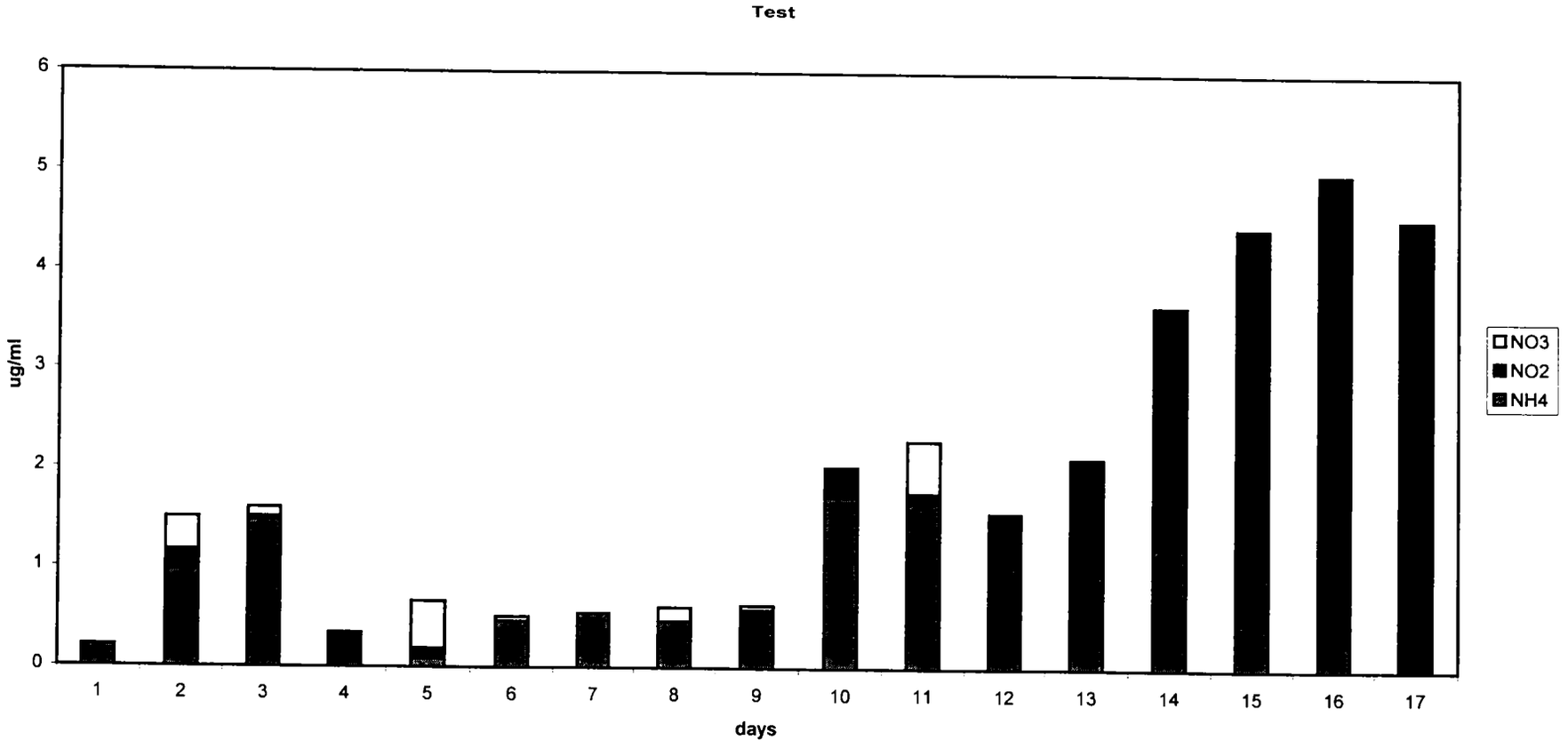


Fig 4 .13 Levels of NH_4^+ - N , NO_2^- - N and NO_3 -N in the larval rearing system with the bioreactors deployed

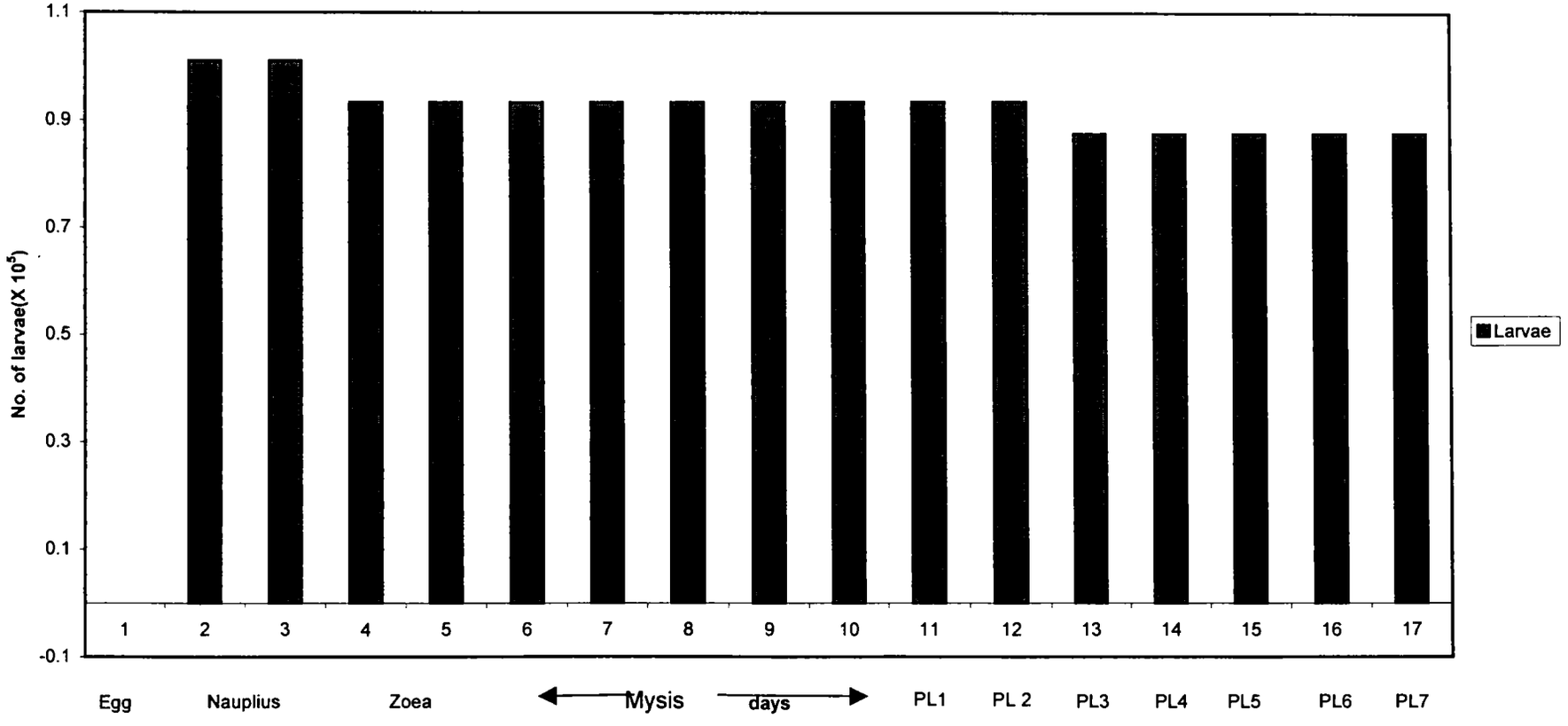


Fig 4.14 Number of larvae survived through various stages during the larval rearing period in the larval rearing system with the bioreactors deployed

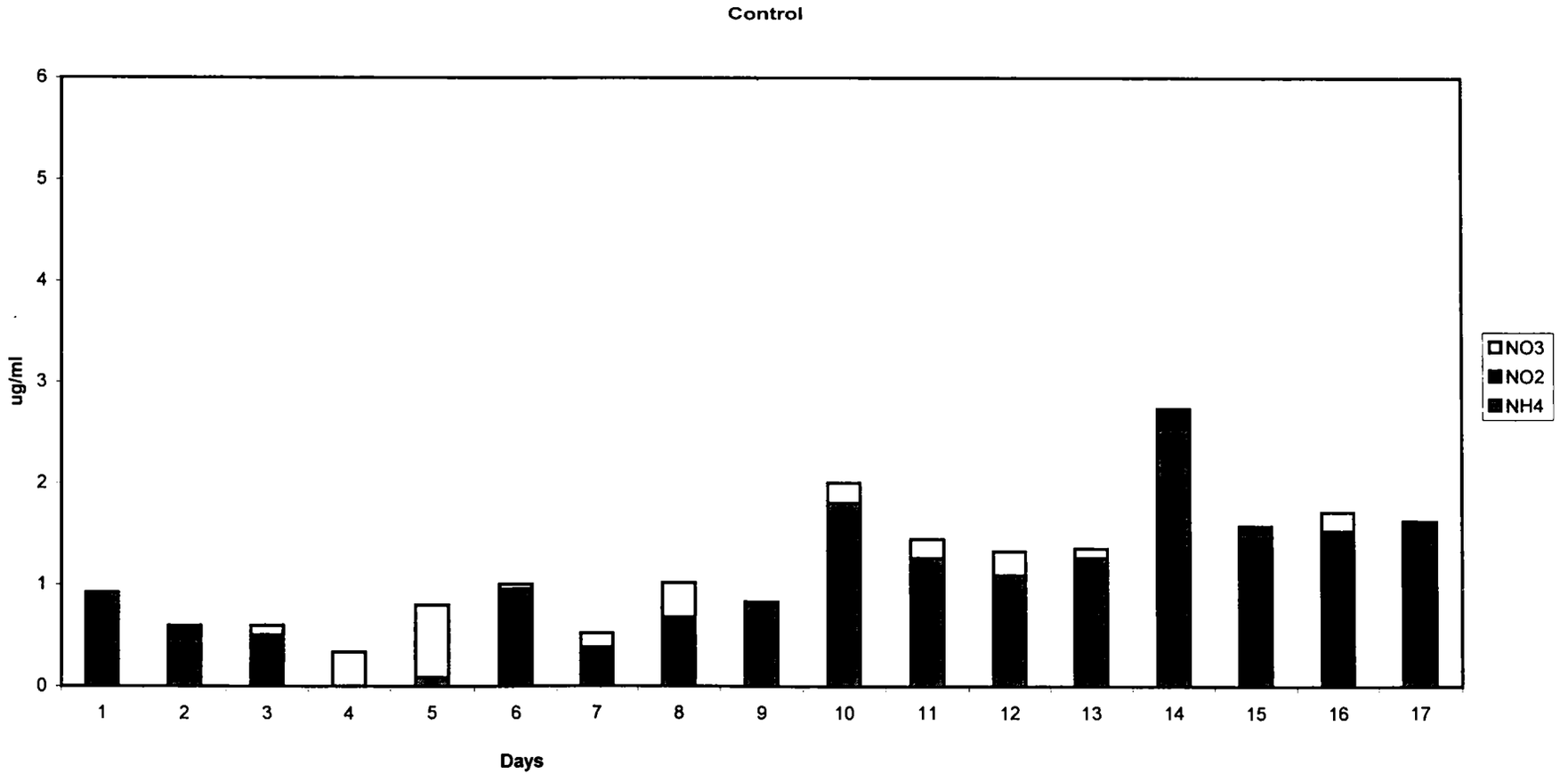


Fig 4. 15 Levels of NH_4^+ - N , NO_2^- - N and NO_3 -N in the larval rearing system without the bioreactors deployed

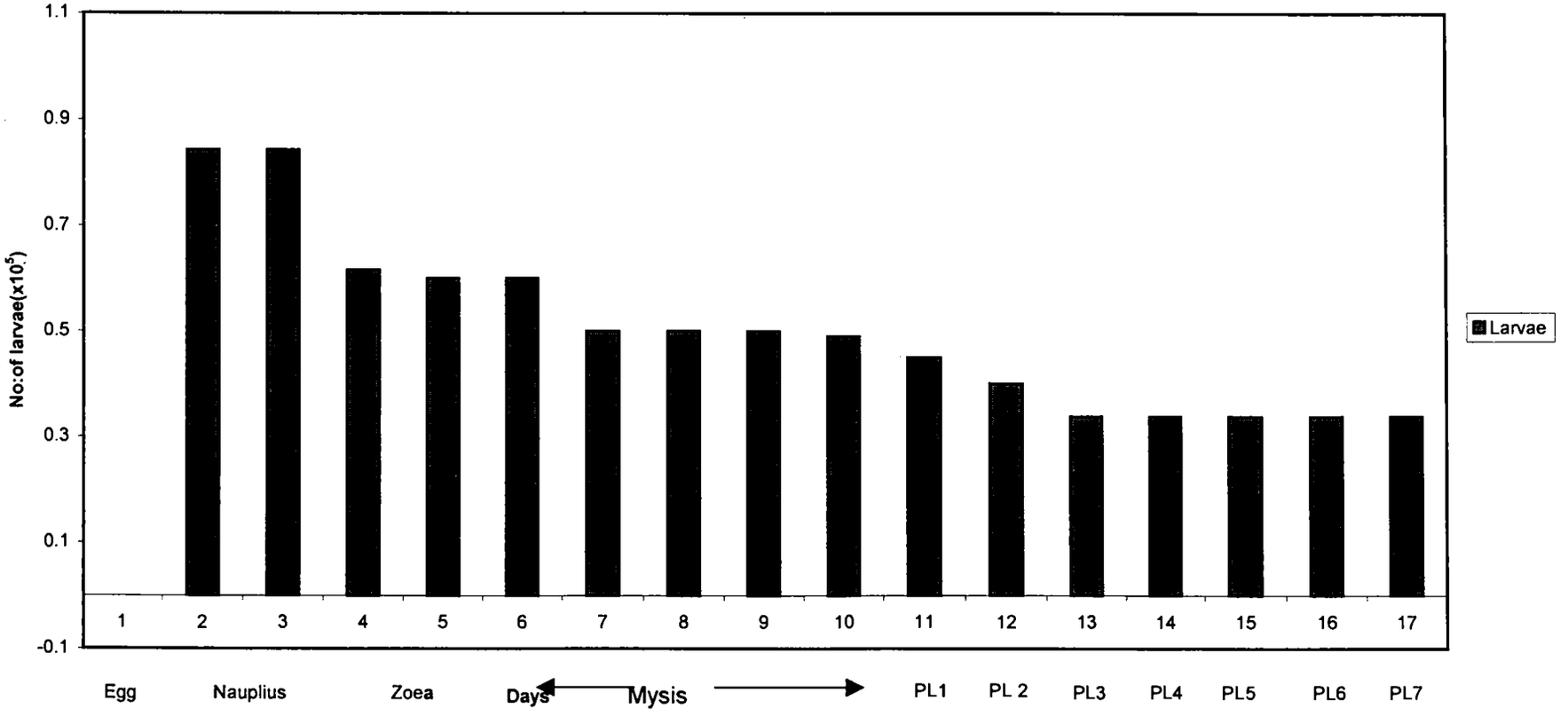


Fig 4. 16 Number of larvae survived through various stages during the larval rearing period in the larval rearing system without the bioreactors deployed

CHAPTER 5

CONCLUSION

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CONCLUSION

Ammonia toxicity is the major limiting factor in penaeid and non-penaeid hatchery systems causing lethal and sublethal effects on larvae depending on the pH values. It has been a pressing need of the aquaculture industry to have a user friendly and economically viable technology for the removal of ammonia, which can be easily integrated to the existing hatchery designs without any major changes or modifications. Only option available now is to have biological filters through which water can be circulated for the oxidation of ammonia to nitrate through nitrite by a group of chemolithotrophs known as nitrifying bacteria. These biological filters are with several inherent drawbacks and short comings as it requires prolonged (1-2 months) conditioning period, entrap feed and larvae, get clogged and becomes too difficult to disinfect the hatchery system by having the biological filter connected, as the process washes away the filter bacteria. In this context, nitrifying bioreactors were conceptualized which would satisfy all requirements of the existing penaeid and non-penaeid hatchery technology and can be easily integrated into the hatchery designs. Two types of bioreactors have been designed and developed. The first category named as *in situ* stringed bed suspended bioreactor (SBSBR) was designed for use in the larval rearing tanks to remove ammonia and nitrite during larval rearing on a continuous basis, and the other to be used for nitrifying freshly collected seawater and spent water named as *ex situ* packed bed bioreactor (PBBR). Basically both the categories of reactors have three main components viz.,

1. Nitrifying consortia
2. Appropriate support material for the nitrifiers and
3. Shell of the reactor

The first one serves as the 'software' of the technology, the second and third ones function as 'hardware'. The following pages summarize how these three components have been developed, assembled, activated and assessment of performance done for active integration to the hatchery technology.

- For development of active nitrifying consortia the samples obtained from different locations were subjected for pre-enrichment in a biological filter set up. The nitrifying consortia developed from the filterent grains of such biological filters were the most potent ones. By this way of pre-enrichment, the nitrifiers which were with the inherent property of attachment to solid substratum, could be isolated and made use of in the development of the bioreactors.

- By a series of primary, secondary and tertiary enrichment procedures 14 nitrifying consortia could be developed which formed the broad base for the nitrifying bioreactors developed. From these, one ammonia and nitrite oxidizing consortium each named as AMOPCU 1, and NIOPCU 1 for penaeid hatcheries, one ammonia and nitrite oxidizing consortium each for non-penaeid hatchery systems named as AMONPCU 1 and NIONPCU 1 were segregated for further use.

- These consortia were amplified in a fermentor employing the medium of Watson (1965) which consisted of seawater at 30 or 15 ppt supplemented with 10 $\mu g\ mL^{-1}$ NH_4^+-N / NO_2^--N and 2 $\mu g\ mL^{-1}$ PO_4-P at a temperature 28^0C with constant stirring at 10 rpm and air passage of 0.6 L min^{-1}. On attaining stationary phase the amplification was terminated, consortia harvested, concentrated by centrifugation, total biomass determined and maintained at 4^0C in a refrigerator. This formed the stock for all further experiments.

- The Gram stained preparations of nitrifying bacterial consortia revealed the presence of cells embedded in glycocalyx, a product of bacteria itself composed of polysaccharide. Along with the majority of Gram negative rods, Gram positive bacteria also could be seen indicating that they were mixed culture consortia. These consortia were not resolved, instead, were used as such, considering that each could be used as separate entities.

- Optimum growth requirements of the four consortia developed were determined, and they were as follows: AMOPCU 1:pH 7.5, temperature 28^0C, substrate concentration 10 μg mL^{-1} and salinity 30 ppt; NIOPCU 1: pH 7, temperature 37^0C, substrate concentration 10 μg mL^{-1} and salinity 25 ppt; AMONPCU 1; pH 8.5, temperature 28^0C, substrate concentration 10 μg mL^{-1} and salinity 10 ppt NIONPCU 1: pH 4.0, temperature 45^0C, and substrate concentration 10 μg mL^{-1} and salinity 5 ppt. The consortium NIONPCU 1 was not found to be suitable for prawn larval rearing system and therefore NIOPCU 1, the nitrite oxidizing consortium designed for penaeid system was acclimatized by lowering the salinity preference to 15 ppt and used for all further work after naming it NIONPCU 1a.

- Unit nitrifying activity (UNA) of the consortia was determined and they varied very much justifying the need for such quantitative assessments of the capability.

- Growth kinetics of all four consortia were determined in fermentor under optimum growth conditions. All the four consortia were more or less equally efficient capable to convert > 87 % of the substrate to the nitrification products suggesting that they were with lesser heterotrophs, a character desirable for nitrifying consortia.

- One of the problems to be addressed with regard to the mass production of nitrifying consortia in fermentor is the tremendous wall growth. Some easy and non-destructive methods should be developed to remove the consortia from the fermentor vessel at the end of the culture period.

- On determining the cell number of nitrifiers in the consortia, it was observed that between consortia differences in cell number of nitrifiers was quite marginal affirming the suitability of culture conditions provided in the fermentor. Also the data suggests that all consortia were harvested at the right moment before the augmented growth of heterotrophs which might happen at the expense of nitrifiers.

- To compensate the lowering of pH and to bring it back to optimum during the growth of ammonia oxidizing consortia such as AMOPCU 1 and AMONPCU 1, 94.0 and 52.25 mL each of 10% sodium carbonate was estimated to be required. This adds to the cost of production at commercial level.

- Compared to the earlier studies yield coefficient of all the consortia were much higher, which can be highlighted as the characteristic of tropical cultures. Here also the yield coefficients, when worked out based on the biomass output were lower except in the case of AMONPCU 1. This again suggests the negative impact of wall growth of consortia.

- Generation time of the consortia ranged from 10.5 - 22.34 hours.

- Specific growth rate of the consortia ranged from 0.033-0.063 L^{-1}, which was very low, compared to the heterotrophs. The precise reason for the low specific growth rate and cell yield is the small energy gain obtained from the oxidation of ammonia and nitrite.

- On phase contrast and scanning electron microscopy observations, the cells of the consortia were found to be embedded in mucilaginous sheath known as glycocalyx.

- Polyethylene imine treatment of plastic beads as an immobilization enhancer was not effective as a certain level of toxicity was seen on nitrifiers. Meanwhile freely suspended cells exhibited profound nitrification, and in the presence of plastic beads also the nitrification was found to go on unhampered. All these observations suggested that an alternative method of immobilization of nitrifiers on bead surface had to be evolved.

- On evaluating the three hour contact of the plastic bead with the nitrifiers for attaining irreversible adsorption of cells, it was found that the duration had to be enhanced considerably. Moreover it was also convinced that the immobilization of the cells had to be carried out under optimum growth conditions using the medium according to Watson (1965).

- By a series of experiments the minimum time required for effective irreversible adsorption of the cells on the plastic surface was found to be 72 hours under optimum growth conditions.

- On exposing the beads made out of 8 different kinds of plastics to the consortium NIOPCU 1, different level of adsorption, characterized by varying level of activity could be obtained. The observation led to the screening of all eight types of plastics against each nitrifying consortium.

- On the basis of the extent of activity observed for each type of plastic with each consortium and also on the basis of cost of the type of plastic and easiness to mould, the plastic coded as PE was segregated for AMOPCU 1 and AMONPCU 1 and the type of plastic coded as LB was segregated for NIOPCU 1 and NIONPCU 1a for further use.

- The plastic beads designed and moulded have a surface area of 0.785 centimeters. To increase the roughness and thereby to enhance adhesion, the surface is sparked. One of the outstanding features of the immobilization technology developed here is that it is an extremely simple process which does not involve the addition of any adsorption enhancers. Instead, the consortium harvested from the fermentor was used as such without any concentration of cells by centrifugation. During immobilization the optimum *growth conditions are provided.*

- Using the above plastic beads as the support material for the attachment of nitrifying consortium a stringed bed suspended bioreactor (SBSBR) has been designed in such a way that if larvae enter the reactor it can easily come out without getting mutilated. The cartridges are easy to be pulled out, washed and replaced.

- The reactor was activated in a specially designed activation mode and could be deployed in the hatchery system without any loss or activity or experiencing any lag.

- On carrying out a field trial with activated SBSBR, NH_4^+-N / NO_2^--N could be brought down along with the increase in the larval survival and metamorphosis.

- The SBSBR developed has been found to be very much user friendly and what the hatchery personnel has to do is to connect the reactor after activation to any of the existing air supply tubing and adjust the volume of air to 1 litre per minute.

- For nitrifying freshly collected seawater before use for larval rearing and for treating the spent water after larval rearing an *ex situ* packed bed bioreactor has been designed, fabricated and activated. To facilitate any integration to

the existing hatchery facility the technology is offered as a package along with the design for water storage and with a recirculation system.

- On employing the two reactors together, both penaeid and non-penaeid larval rearing systems can be made a closed recirculating system at least for a season.

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